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UNIVERSITY OF ALBERTA

Enzyme-Linked Immunosorbent Assays for Sulfonamides

by

Hasmukh B. Sheth

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Fall 1991

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Date: ....., 1991

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis

entitled **Enzyme-Linked Immunosorbent Assays for Sulfonamides**

submitted by **Hasmukh B. Sheth**

in partial fulfillment of the requirements for the

degree of **Doctor of Philosophy**

in **Food Chemistry**

Peter Sporns
Dr. P. Sporns (Supervisor)

L. Ozimek
Dr. L. Ozimek (Food Science, University of Alberta)

M. Palcic
Dr. M.M. Palcic (Food Science, University of Alberta)

Bhagirath Singh
Dr. B. Singh (Immunology, University of Alberta)

Michael E. Stiles
Dr. M.E. Stiles (Food Science, University of Alberta)

R.Y. Yada
Dr. R. Yada (Food Science, University of Guelph)

Date: June 17, 1991

DEDICATION

To My Beloved Parents

Abstract

Studies with sulfathiazole (ST) as a representative sulfonamide demonstrated that N^4 aromatic amino group of sulfonamides can react with reducing sugars to form a variety of different sugar-bound compounds. ST was not destroyed in this reaction, and free ST could be produced from some of these sugar-bound compounds by aqueous dilution and especially, by acidification. Different effects of these sugar-bound ST compounds, especially the N^4 linked ST Amadofi compound, were noted on various methods of sulfonamide residue analysis. The sugar-bound ST compounds exhibited chromatographic behaviour different from that of free ST, did not visualize with a common TLC Bratton-Marshall spray specific for sulfonamides, did not inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* strains susceptible to free ST, but did inhibit binding of antisera in an indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA) as much as 33 times greater, on a molar basis, than free ST.

A simple Enzyme Immunoassay (EIA) was developed to screen honey samples for ST adulteration. Honey samples required only a 30-fold dilution before use in the procedure. Because 96-well microtiter plates were used and only 100 µL of diluted honey sample was required per well, numerous replicates or samples could be tested simultaneously. The EIA was able to detect at least 0.3 ppm levels of ST in honey and also provide a rough quantitation of ST amounts.

Sulanilamide derivatives were chemically linked to proteins in such a way that the aromatic amino group, common to all sulfonamides, was distal to the protein. Subset of antibodies developed against these immunization conjugates could be used competitively with different sulfonamide haptens, linking methods and proteins to develop ELISA methods that had a broad spectrum of sulfonamide antibiotic recognition. Using optimal ELISA protocol, the presence of eleven structurally different in one case and fourteen structurally different sulfonamides in other was detected. The sulfonamides recognized by

competitive ELISA had similar steric characteristics but considerable variation in electronic configuration.

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I am grateful to my supervisor, Dr. Peter Sporns for providing me with the opportunity to carry out this research. I am indebted to him for his continued professional and personal guidance throughout this program.

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List of Abbreviations

B-M	Bratton-Marshall
BSA	Bovine Serum Albumin
C.V.	Coefficient of Variation
DCU	Dicyclohexyl Urea
DMF	Dimethylformamide
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
FD&C	Food, Drug and Cosmetic
GC	Gas Chromatography
GC-MS	Gas Chromatographic-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LPH	*limulus polyphemus hemolymph*
MH	Mueller Hinton
MNL	Mononuclear Leukocyte
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
OV	Ovalbumin
PABA	*p*-aminobenzoic acid
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween-20
ST	Sulfathiazole
TLC	Thin-Layer Chromatography
UV	Ultraviolet

1. INTRODUCTION

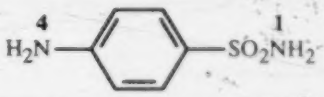
1.1 Historical Development of Sulfonamides

Spink (1943), Lawrence (1946) and Sophian *et al.* (1952) have described the history of sulfonamides. From these manuscripts some of the important developments are summarized below.

Sulfonamides are a group of synthetic sulfanilamide antibiotic derivatives. In 1908, the first sulfonamide, sulfanilamide was synthesized by a German scientist, Gelmo. Though this drug was used in the dye industry for 25 years to form azo-dyes, its bacteriostatic properties were unknown. In 1932 Mietzsch and Klarer patented the sulfanilamide azo-dye derivative, prontosil for its antibiotic properties. It was shown by Dogmak that this antibiotic was effective against lethal doses of streptococci in mice. When prontosil and sulfanilamide were shown to have shortcomings in the therapy of bacterial infections, efforts were made to synthesize new derivatives of sulfanilamide. Dohrn and Diedrich reported the synthesis of sulfacetamide in 1938. In 1939, Landon Sjogren synthesized ST and in the same year American Cyanamide Company announced the synthesis of sulfadiazine. Robin *et al.* in 1940 synthesized sulfadiazine and sulfamerazine. In the same year, sulfaguanidine was synthesized by Winnek. After these pioneering developments hundreds of sulfanilamide derivatives have been prepared.

1.2 Chemistry of Sulfonamides

The structure of sulfanilamide is given below.



Sulfonamides or sulfanilamide derivatives are generally white to yellowish powdery compounds. These compounds can form neutral alkali salts because of the acidity of the hydrogen on N^1. Sulfonamides in free acid form are sparingly soluble in water, while their

sodium salts are freely soluble. Sulfonamides are readily soluble in alkaline solution in which they act as weak acids. The *p*-amino group at N^4 is very weakly basic. It is well known that if this amino group is substituted by other groups or moved to the *m* or *o* position, the resulting compounds have substantially lower activity. On the other hand, if different R groups are linked through the N^1 position, potent sulfonamides are obtained. Because of this, most sulfonamides are N^1 substituted derivatives of sulfanilamide. Figure 1.1 shows the different R groups for sulfonamides.

1.3 Structural Activity Relationship of Sulfonamides

The N^4-amino group and the sulfonamide group ($-SO_2NH_2$) are essential for a sulfonamide to be active. Usually inactive sulfonamides are obtained if there is substitution in the benzene ring. As mentioned above, replacement or movement of N^4 amino group yields less active compounds, while linking of different R groups through the N^1 position, results in compounds with various activities. Generally carbo-aromatic and N^1-alkyl substitution in the basic sulfanilamide structure results in lowering or loss of activity. Alternatively, N^1-acyl substitutions leads to compounds with improved activity. It was shown by Bell and Roblin (1942) that the higher the electronegativity of SO_2 group at the N^1 position, the higher the bacteriostatic activity of sulfonamide. Numerous sulfonamide analogs with five and six membered heterocyclic rings have been synthesized. Positioning of substituents on the hetero ring also has a significant effect on the activity of resulting sulfonamide analogue. Many five membered heterocyclic sulfonamides contain one or more methyl groups. The addition of these methyl groups increases the hydrophobicity and therefore increases their protein binding and folate-enzyme binding properties. Sulfapyridine, sulfapyrimidines, sulfapyridazines are sulfonamides with six membered heterocyclic R groups.

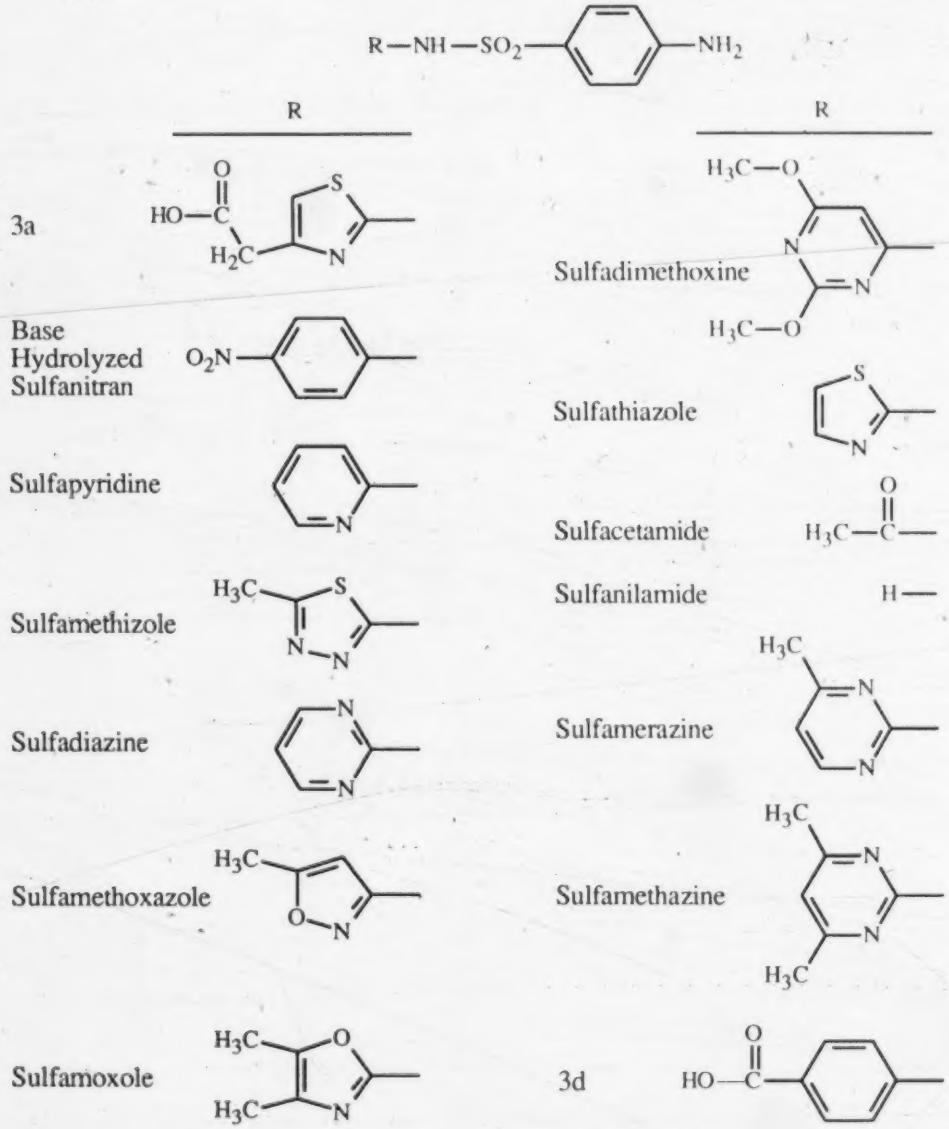


Figure 1.1. Structures for common sulfonamides.
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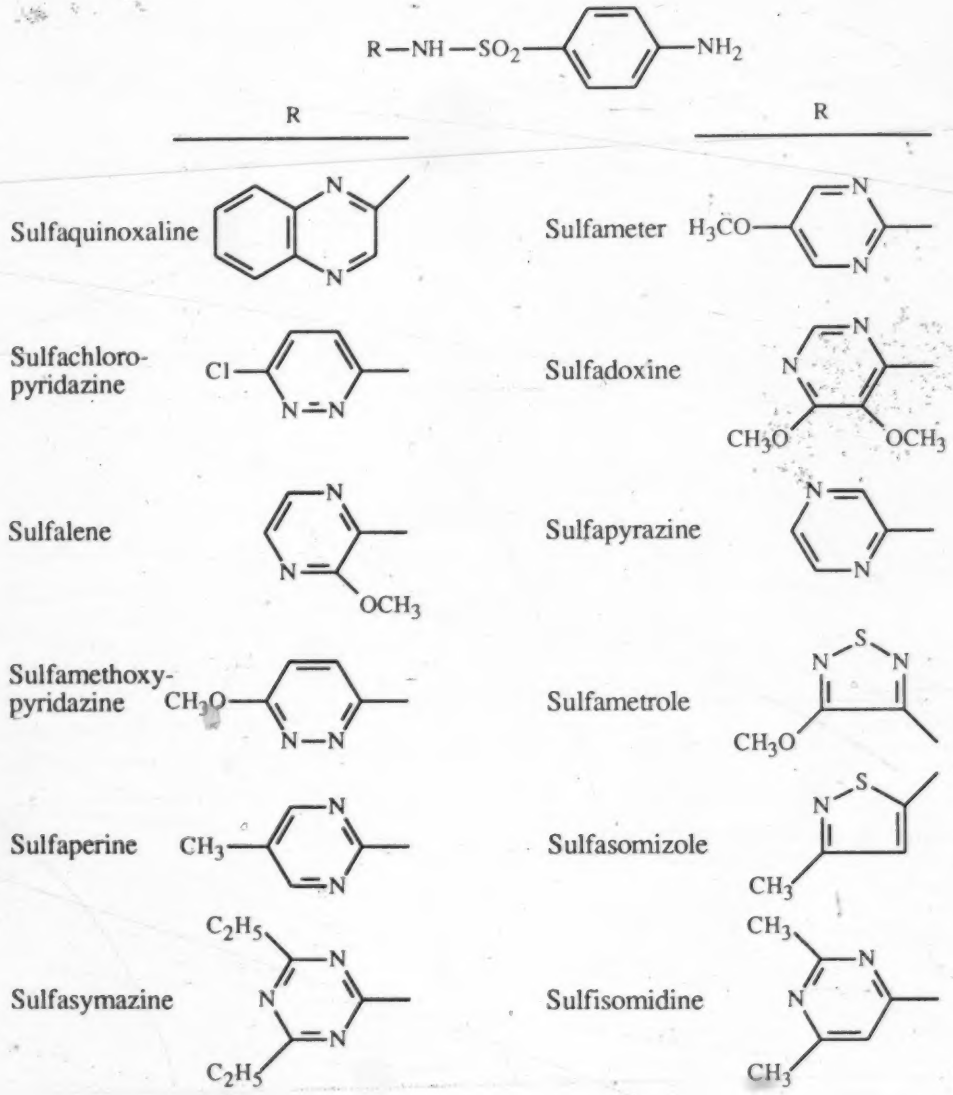


Figure 1.1 *(...continued).* **Structures for common sulfonamides.**

1.4 Mode of Action of Sulfonamides

Sulfonamides are bacteriostatic compounds. The mechanism by which they act is clearly understood at the enzyme level. The developmental history of the mode of action of sulfonamides is described by Anand (1975).

It is well known that molecules such as purine nucleotides, thymidylate, serine and methionine are essential for the survival of the cell. Figure 1.2 shows the synthetic pathway of these compounds. The main metabolite necessary in the synthesis of these molecules is tetrahydrofolate. The synthesis of tetrahydrofolate is initiated by the condensation of PABA with dihydropteridine. PABA and most sulfonamides (with unblocked N^4-amino group) display considerable structural similarity and therefore these sulfonamides can block the synthesis of tetrahydrofolate. It was shown by Miller (1944) and Miller *et al.* (1947) that sulfonamides can inhibit folic acid synthesis in *E.coli*. Nimmo-Smith *et al.* (1948) and Lascelles and Woods (1952) showed the inhibition of folic acid synthesis by sulfonamides and its competitive reversal by PABA. If a sulfonamide was incubated with dihydropteridine the product formed is a sulfonamide analog of dihydropteroate.

Animal cells which require preformed folic acid are not affected by sulfonamides. Folic acid in blood and tissues is usually linked to polyglutamate and proteins. This conjugated form of folic acid can not be used by bacteria and therefore only bacteria are adversely affected by sulfonamides and their growth is inhibited.

1.5 Use of Antibiotics in Agriculture

In 1950's the United States Food and Drug Administration approved the use of antibiotics as feed additives. Antibacterial agents including sulfonamides are used at subtherapeutic concentrations (Gustafson, 1986). These drugs are used to prevent infections caused by bacteria and protozoa and to increase the rate of weight gain. Sulfonamides are used in ural practice alone or in combination with other antibiotics. Use of these ave enhanced animal growth and increased food supplies

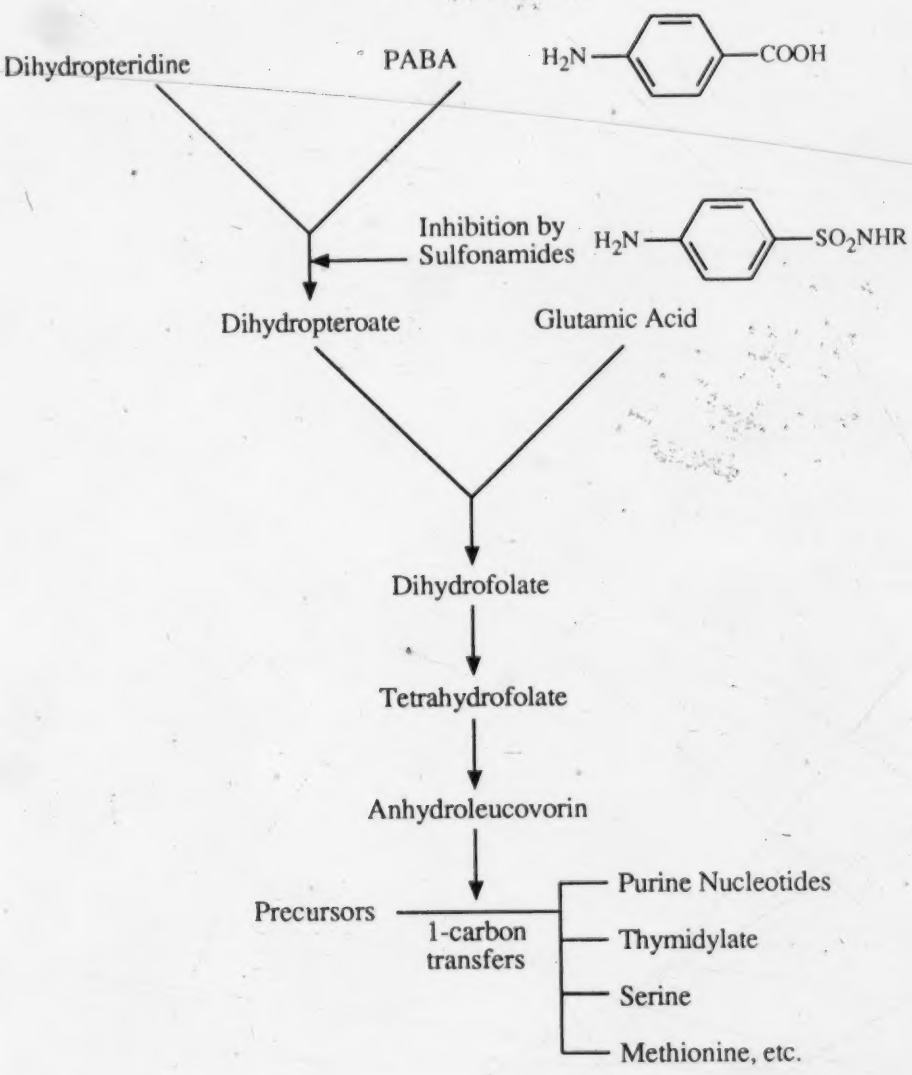


Figure 1.2. Enzymatic Synthesis of Tetrahydrofolate (adapted from Anand, 1975).

(Nichols and Keys, 1984). Sales of animal feed additives was worth more than 1.1 billion dollars in 1983 (Franco *et al.*, 1990) and according to Tindall (1985) 50% of the 35 million pounds of antibiotics manufactured in the United States were fed to animals. Use of antibiotics as feed additives in the production of beef, pork and poultry alone has saved consumers 3.5 billion dollars annually in the United States (Marshall, 1980).

Franco *et al.* (1990) suggest that antibiotics improve feed efficiency and growth by selectively inhibiting nutrient using organisms, increasing feed and water intake, inhibiting toxin producing organisms and improving the absorption of nutrients such as vitamins, minerals and amino acids. Sulfamethazine, also called sulphadimidine is used in combination with chlortetracycline in swine and in cattle for enhancing weight gains and feed efficiency.

Sulfonamides are also used for controlling diseases. For example, honey bee larvae are susceptible to American foulbrood (Argauer, 1986), a disease caused by the organism, *Bacillus larvae*, which can destroy a hive. ST, a stable antibiotic has been used to control this disease. In chickens coccidiosis is caused by *Eimeria tenella* (Mitrovic *et al.*, 1969). Sulfaquinoxaline alone or sulfadimethoxine in combination with ormetoprim is used to control these diseases. The same treatment can be used to prevent coccidiosis in turkeys. Sulfonamides are also useful for treating mastitis in cows.

1.6 Untoward Reactions to Sulfonamides

Numerous adverse reactions are experienced after the administration of sulfonamides to humans and animals. Weinstein *et al.* (1960) reported that the occurrence of these type of reactions is approximately 5% in humans. There are number of reports of toxic reactions to sulfonamides in humans and dogs (Scott *et al.*, 1976; Toth and Derwelis, 1980; Anderson *et al.*, 1984; Davies, 1984; Giger *et al.*, 1985; Mandell and Sande, 1990; Nesbitt, 1986; Grondalen, 1987; Harvey, 1987; Cribb and Spielberg, 1990; Cribb *et al.*, 1990).

The clinical manifestations of sulfonamide toxic reactions in human are the urinary tract disease crystalluria, fever, skin rash, and multiorgan toxicity in the liver, kidney, bone marrow, heart or thyroid gland. Goiter production has also been reported in sulfonamide therapy. Sulfamethazine is also known to cause cancer of the thyroid gland (Littlefield *et al.*, 1990).

In the process of toxicity development, the parent drug is converted into a reactive metabolite which may either directly cause cell death or bind to cellular proteins and induce an immune response (Park *et al.*, 1987). This process is dependent on the immune responsiveness of the host.

In many instances a drug needs to be metabolically activated and the adverse side reactions are mainly dependent on the balance of generation and detoxification of reactive metabolites. Shear *et al.* (1986) have examined the pathway of sulfonamide metabolism and proposed a role of reactive metabolites in the pathogenesis of adverse reactions. As shown in Figure 1.3 the N^4 amino group of sulfonamide is acetylated. The acetylated sulfonamide is a non-toxic metabolite. Acetylation of the *p*-amino group is mediated by the enzyme N-acetyl transferase. In humans, approximately 50% of the population are slow acetylators and 50% are fast (Weber and Hein, 1985). In one study (Shear *et al.*, 1986) it was found that six out of six human patients experiencing sulfonamide hypersensitivity were slow acetylators. The N^4 amino group can also be oxidized to a hydroxylamine. Slow acetylation of the free amino group of sulfonamide can result in the production of more hydroxylamine which can cause cellular toxicity as shown in Figure 1.3. Spielberg (1980) has reported an *in vitro* MNL toxicity assay. A correlation between susceptibility of MNL to the untoward effects of hydroxylamine metabolites and susceptibility of sulfonamide toxicity has been shown by Rieder *et al.* (1988). Cribb *et al.* (1990) have reported that N^4 oxidation of sulfonamides can also form nitroso metabolites which can be involved in the pathogenesis

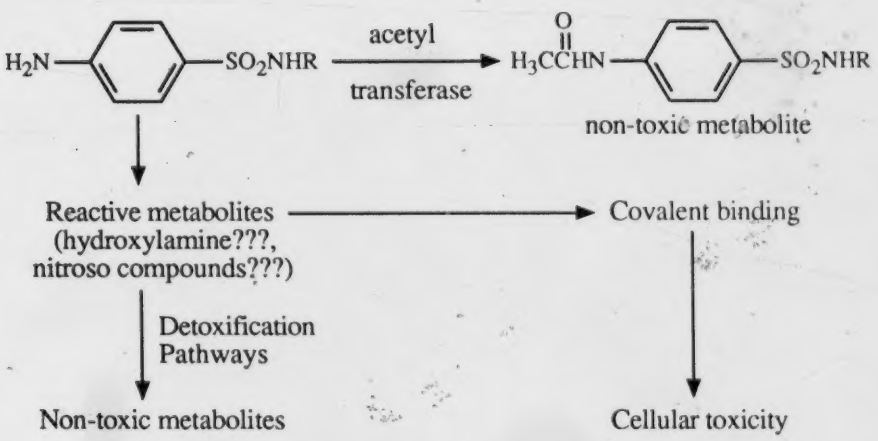


Figure 1.3. Pathway of Sulfonamide Metabolism and Proposed role of Reactive Metabolites in the Pathogenesis of Toxic Reactions (adapted from Shear *et al.*, 1986).

of toxic reactions of sulfonamides. From these observations it is currently believed that slow acetylation of the *p*-amino group and reduced capacity of an individual to detoxify hydroxylamine and nitroso metabolites can lead to the toxic effects of sulfonamides.

1.7 Acquired Bacterial Resistance to Sulfonamides

A danger of using sulfonamides, especially as feed additives, is the development of antibiotic-resistant strains. Mandell and Sande (1990) reported that when this resistance is fully developed it is usually persistant and irreversible. This resistance can be developed by random mutation and selection or by the transfer of resistance by plasmids. Many factors such as: (1) overproduction of essential metabolites such as PABA (Landy *et al.*, 1943) or dihydropteridine; (2) antibiotic antagonists which can make sulfonamide inactive; (3) an altered sensitivity of enzymes inhibited by sulfonamide action (Wolf and Hotchkiss, 1963; Pato and Brown, 1963); (4) an alternative metabolic pathway for synthesis of an essential metabolites; or (5) altered permeability of the organisms to sulfonamides, can be responsible for the antibiotic-resistance. Wise and Abou-Donia (1975) discovered that genes, responsible for sulfonamide resistance, code for a metabolite dihydropteroate synthase which is not affected by sulfonamides.

Mobile elements in bacterial cells, also called transposons, can move from one site to another on the genome and be transferred into another cell. Plasmids can contain resistance for multiple antibiotics and therefore the mobility of transposons can enhance the antibiotic resistance capability of bacteria. R plasmids found in members of the *Enterobacteriaceae* carry sulfonamide resistance genes (Guerineau, 1990). Yamamoto *et al.* (1981) and De La Cruz and Grinstead (1982) have studied the transposons found in R plasmids of *Enterobacteriaceae*. *Salmonella typhimurium* contains a plasmid responsible for resistances to sulfonamides, ampicillin, tetracyclin and streptomycin (Datta and Kontomichalou, 1965). This plasmid is also responsible for resistances to compounds such as arsenite, arsenate and antimony compounds (Silver *et al.*, 1981)

Foods derived from animals treated with sulfonamides are likely to contain antibiotic-resistant bacteria and food products such as milk, eggs and meat provide very good culture media for the growth of these bacteria (Franco *et al.*, 1990). Unhygienic slaughter conditions, inadequate refrigeration and poor processing and cooking methods can increase the contamination process. Once this poorly handled food is consumed, sulfonamide resistant bacteria can colonize the gut and transfer resistance to other bacteria in the intestine by plasmid or transposon transfer.

1.8 Analytical Methods for Sulfonamides

For more than 50 years sufonamides have been used as antimicrobial agents. The United States Federal FD&C act permits the use of specific sulfonamides in specific species (Horwitz, 1981). FD&C requires the withdrawal of permitted sulfonamides from use long enough to eliminate the residual contamination in foods obtained from these sources. Many sulfonamides, such as ST, are not registered for certain agricultural uses in many countries (Sheth and Sporns, 1990) and therefore the presence of a specific sulfonamide not approved for use in a given species or related food product is a violation of the regulatory act.

In food tissues, sulfonamides are permitted to the level of 0.1 ppm while the allowed limit of sulfadimethoxine in milk is 0.01 ppm. No sulfonamides are allowed in certain species, for example, sulfapyridine in cattle, sulfamerazine in trout and sulfanitran in chickens (Horwitz, 1981). The joint FAO/WHO expert committee (1989) on food additives recommended maximum sulfonamide residue levels in meat, liver, kidney and fat as 0.3 ppm as total residue. In the same products sulfamethazine levels were fixed at 0.1 ppm. The same committee also recommended maximum sulfonamide residue levels in milk of 0.05 ppm as total residue and sulfamethazine levels of 0.025 ppm. In Canada the permitted sulfonamide limit without specific regulations is 0.1 ppm.

For monitoring and enforcing the tolerance levels of these antibiotics, several methods are available to the analyst. Horwitz (1981) has reviewed the analytical methods for sulfonamides. Analytical methods available for identification and quantitation of sulfonamides include: (1) simple chemical methods such as colorimetric (Bratton and Marshall, 1939; Bregha-Morris, 1979); (2) microbiological methods such as microbial inhibition tests (Read *et al.*, 1971) and microbial receptor assays (Charm and Chi, 1988); (3) chromatographic methods such as TLC (Bregha-Morris, 1979; Neidert *et al.*, 1986; Thomas *et al.*, 1981; Thomas *et al.*, 1983; Wyhowski de Bukanski *et al.*, 1988; Unruh, *et al.*, 1990), liquid chromatography (Belliardo, 1981; Parks, 1982; Argauer *et al.*, 1982; Smallidge *et al.*, 1988; Conway, 1988; Weber and Smedley, 1989; Long *et al.*, 1989; Ahmed and El-Gizawy, 1989; Long *et al.*, 1990; Long *et al.*, 1990; Agrawal, 1990; Smedely and Weber, 1990; Horii *et al.*, 1990; Long *et al.*, 1990) and GC (Fravolini and Begliomini, 1969; Nose *et al.*, 1976; Goodspeed *et al.*, 1978; Manuel and Steller, 1981; Munns and Roybal, 1982); (4) spectrophotometric methods such as those using MS (Suhre *et al.*, 1981; Stout *et al.*, 1984; Simpson *et al.*, 1985; Matusik *et al.*, 1987; Takatsuki and Kikuchi, 1990; Matusik *et al.*, 1990) and (5) immunological methods (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; Singh *et al.*, 1989; Dixon-Holland and Katz, 1989; McCaughey *et al.*, 1990; Sheth *et al.*, 1990; Sheth and Sporns, 1990).

None of the methods described above is perfect. Colorimetric methods such as the B-M test detect the aromatic amino group of sulfonamides. Primary aromatic amines, which can be present in body fluids and tissues from normal metabolism, decomposition and autolysis or from other antibiotics used with sulfonamides can interfere with this test.

Microbial methods are expensive, time consuming and are generally qualitative. Shortcomings of one such analytical method (Charm test) are described in section 2.3.2.

If analysis is carried out by methods such as HPLC or GC, the sulfonamide needs to be extracted from the substrate. Sulfonamides are sparingly soluble in non polar solvents but soluble in polar solvents such as ethanol, acetone, acetonitrile, methanol and ammonia.

Crisp (1971) used dimethyl formamide and chloroform and found that the extraction procedure produced extremely stable emulsions. There is no literature available for the comparison of the efficiency of solvents for the extraction of sulfonamides from various substrates (Horwitz, 1981). Different sulfonamides have different solubilities in solvents. When sulfonamides are in aqueous solutions, their extraction by organic solvents is determined by the pH of the solution. It can be difficult to quantitatively transfer sulfonamides from aqueous solutions to a water immiscible organic solvent. A very important part of sulfonamide residue analysis is the cleanup step for the removal of minor components which can interfere in the determinative step. For example, when a chromatographic determination is made, cleanup must be targeted to remove materials with retention times similar to sulfonamides. TLC is a relatively quick method of sulfonamide analysis but it is generally used for qualitative purposes. Quantitation of sulfonamides by TLC can be tedious.

MS gives unique fragmentation peaks for compounds and is a very accurate method of sulfonamide determination. This method alone, however, can not quantitate sulfonamides. HPLC and GC methods are very time consuming, expensive and recoveries obtained by HPLC and GC methods are sometimes inconsistent.

GC-MS can give satisfactory results but analysis costs per sample are very high. This is one of the only methods of sulfonamides determination which can be used as a confirmatory method.

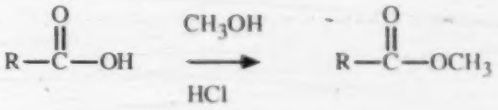
In recent years, immunological methods have become more popular because of their advantages over other methods especially when used as screening methods. Engvall and Perlmann (1971) first reported quantitative ELISA for immunoglobulin G. Immunoassay screening methods for sulfonamides listed above and immunoassay kits for the detection of sulfonamide residues developed by many commercial companies are specific for individual sulfonamides, while the list of sulfonamides of concern in food continues to grow (Long *et al.*, 1989; Matusik *et al.*, 1990; Takatsuki and Kikuchi, 1990). Since immunoassays act as

rapid screening methods for sulfonamides and require further confirmation by some other method (as do all other methods except MS) it would be more efficient if an immunoassay was developed using antibodies that could detect a wide variety of common sulfonamides. Part of this work reports the first attempts to produce such an immunoassay.

2. RESULTS AND DISCUSSION

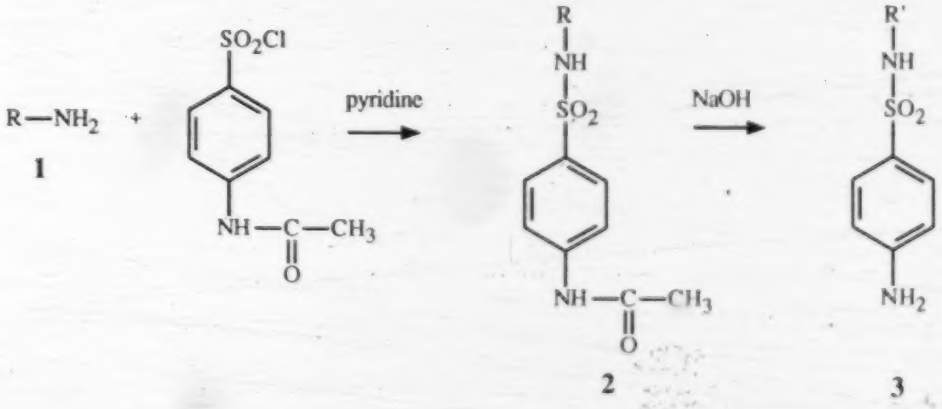
2.1 Synthesis of Different Sulfonamide Analogs

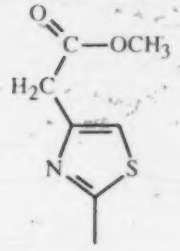
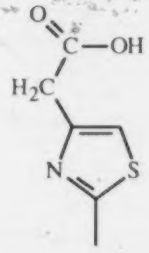
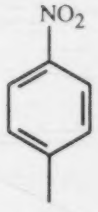
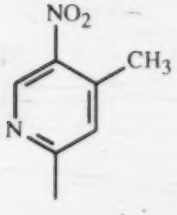
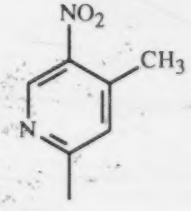
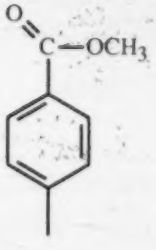
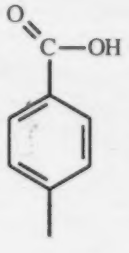
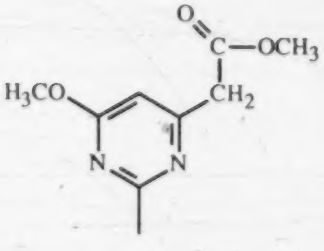
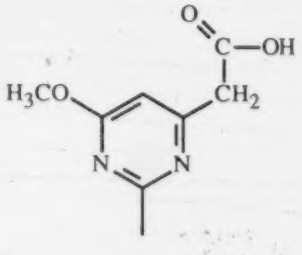
Most sulfonamides have been synthesized by reacting N-acetylsulfanilyl chloride with compounds having a basic amino group. When 2-amino-4-thiazole acetic acid and PABA were reacted with N-acetylsulfanilyl chloride, the desired products were not obtained. This problem could be due to the formation of zwitter ions. To overcome this problem these compounds were converted into corresponding methyl esters as shown below:

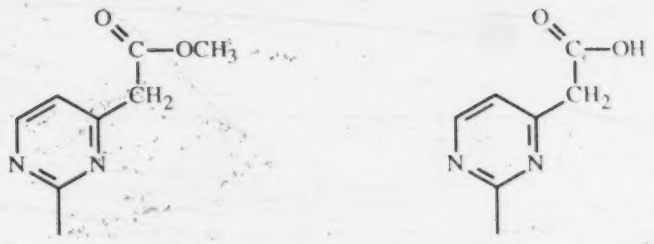


Scheme 1 shows the general synthesis of sulfonamides. When compounds with a basic amino group (1a-1f) were run on a TLC plate using 1:4 methanol:chloroform, R_f values for both starting materials and products (2a-2f) were very close to the solvent front. These R_f values made isolation of products very difficult. N-acetylsulfanilyl chloride did not move from the origin in the 1:4 methanol:chloroform solvent system. Therefore, N-acetyl-sulfanilyl chloride was used in excess with compounds 1a-1f to facilitate later separation of product.

SCHEME 1

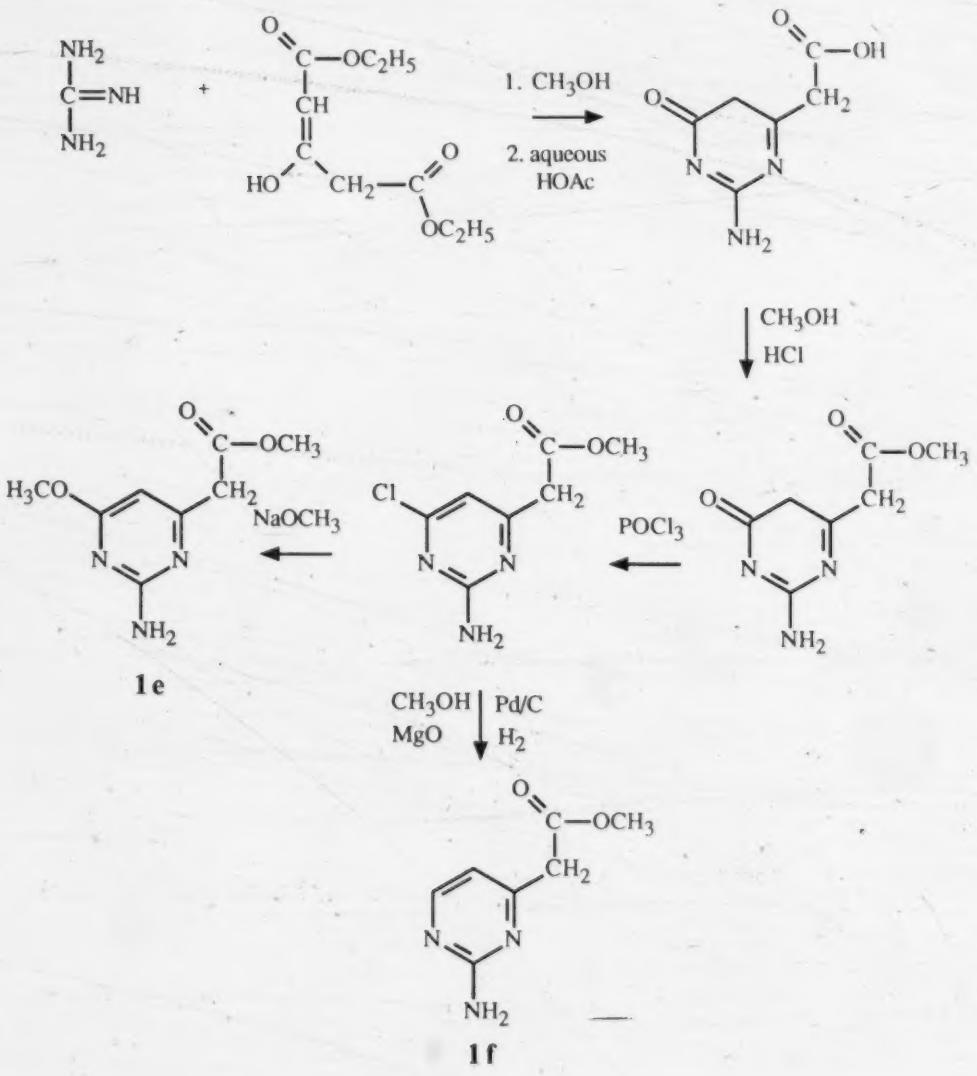


Compound	1 & 2 (R)	3 (R')
a		
b		
c		
d		
e		

f 

Scheme 2 shows the synthesis of compounds 1e and 1f.

SCHEME 2



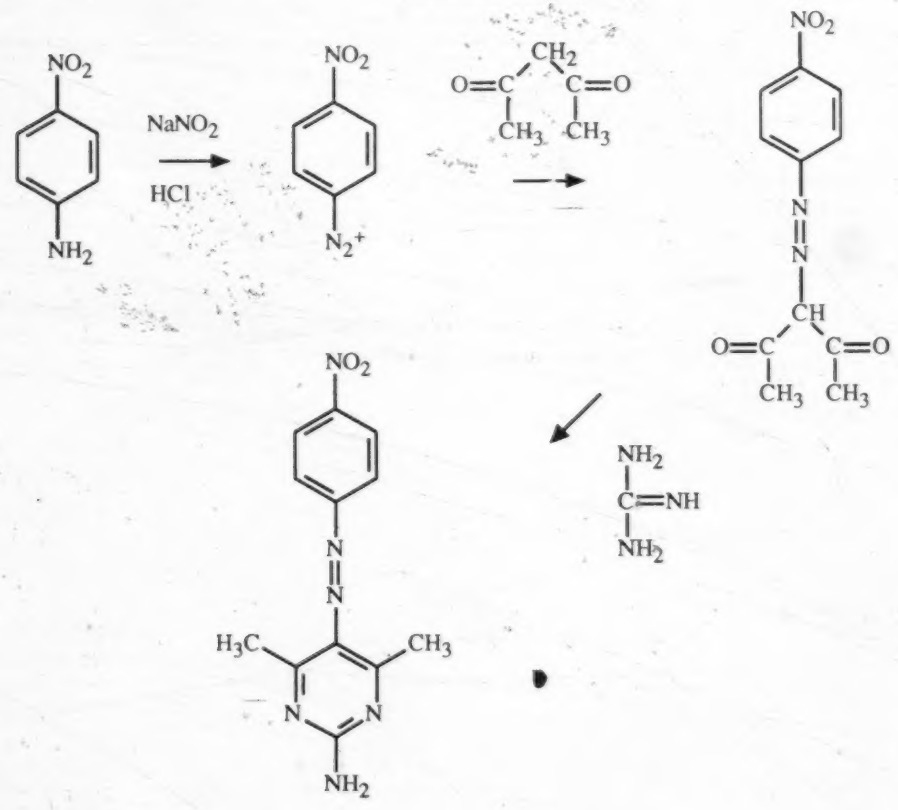
1e

1f

When products 2c and 2d were extracted from aqueous solutions using methylene chloride, highly stable emulsions were obtained. To break these emulsions sodium sulfate was used.

Scheme 3 shows the synthesis of compound 4.

SCHEME 3

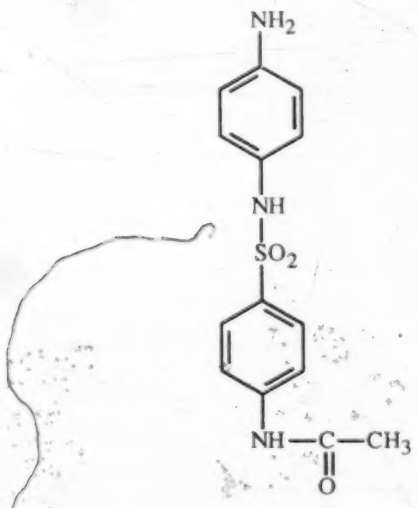


4

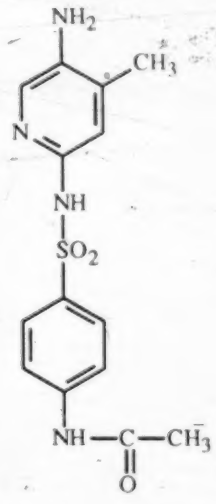
This crystalline material was not soluble in dry pyridine, and most organic solvents. It was only sparingly soluble in hot DMF. After partly dissolving this material in DMF, pyridine was added and condensation with N-acetylsulfanilyl chloride was attempted under rigorous conditions. TLC and MS analyses indicated no formation of sulfonamide analog of compound 4. In another attempt compound 4 was solubilized in hot DMF and the condensation reaction was carried out using the catalyst 4-dimethylamino pyridine. Again

the desired product did not form. Failure of this reaction could be explained by the lack of basicity of the amino group of compound 4.

Reduction of nitro groups of compounds 2b and 2c produced compounds 5 and 6:



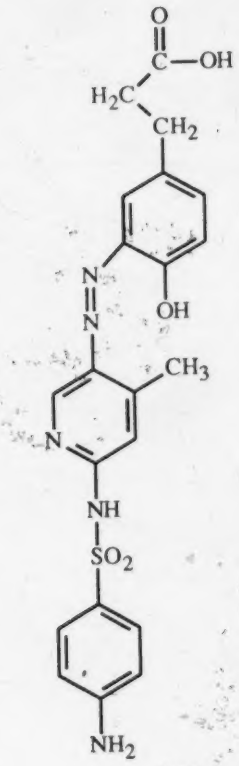
5



6

Use of 1% platinum dioxide did not completely reduce starting materials after 30 hours, as indicated by TLC. To overcome this problem, 100% platinum dioxide on a weight basis was used and after 12 hours it was found by TLC that the starting materials were completely converted into products.

Compound 7 was synthesized from compound 6 and 3-(*p*-hydroxyphenyl) propionic acid by the diazo reaction followed by base hydrolysis. The diazo reaction is described in the synthesis of sulfonamide-protein conjugates (Scheme 4).

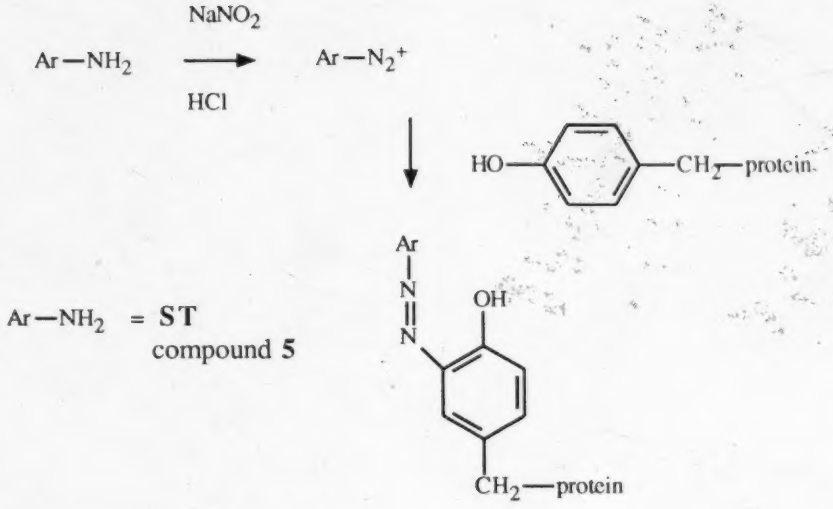


7

2.2 Synthesis of Sulfonamide-Protein conjugates

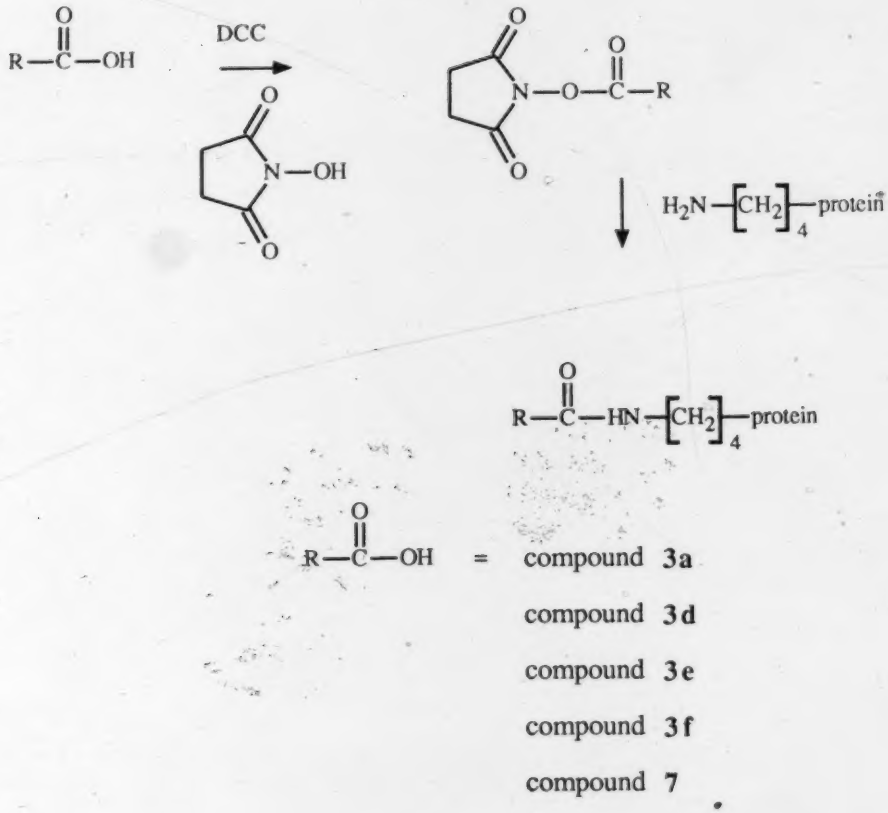
Diazo and N-hydroxysuccinimide active ester methods were used to link different sulfonamide haptens to proteins. ST-LPH, ST-BSA and 5-OV conjugates were prepared by the diazo method (Scheme 4). This was found to be a very convenient method of linking aromatic amino groups to proteins. It was easy to link as many as 44 moles of sulfonamide (ST) per mole of protein (determination of epitope density is described in experimental section 3.11). These conjugates were highly soluble in phosphate buffer.

SCHEME 4



3a-LPH, 3a-BSA, 3d-OV, 3e-LPH, 3f-LPH and 7-LPH conjugates were prepared by N-hydroxysuccinimide active esters (Scheme 5). It was difficult to link a higher number of sulfonamide groups per mole of protein and keep conjugate soluble in phosphate buffer. The maximum number of groups attached per mole of protein in a soluble conjugate were 11. In the active ester reaction DCU was produced. This product was insoluble in aqueous solutions. If a slight amount of DCU is carried forward with protein, it produced insoluble particles. To obtain soluble conjugates, conjugates were centrifuged after dialysis and only the supernatants were used for freeze drying. Conjugates used for immunization were not centrifuged because solubility was not a necessity.

SCHEME 5



2.3 ST analysis

2.3.1 Effect of Reducing Sugars Reacting with ST on Chromatographic and Colorimetric Analysis Methods

To study the reaction between ST and individual reducing sugars, experiments were carried out with large concentrated quantities of the sulfonamide and an approximately three fold excess of the reducing sugars glucose and fructose. The presence of the ST in any reaction material could be detected by UV absorbance after TLC analysis with plates containing a fluorescent indicator, since the monosaccharides had no UV absorbance.

With our TLC development system, free ST had an R_f of 0.76, while a new streak of UV-absorbing material appeared within 2-3 days at R_f 0.09-0.30 for the glucose solution and at R_f 0.11-0.42 for the fructose solution. The slower moving streak of material was shown to contain carbohydrate by the ease with which this material and the monosaccharides charred (glucose and fructose R_fs of 0.07 and 0.16, respectively). Unlike free ST, the slower streak of material did not develop a pink color when sprayed with the B-M spray. After the concentrated glucose and ST solution had stirred at room temperature for 40 days, a 0.5 mL portion of the reaction mixture was removed and the glucose-bound ST (material in slower running streak) was separated from free ST on a silica column. Using UV absorbance at 280 nm in 3.5 N hydrochloric acid solvent, it was found that 32% of the recovered ST was bound to glucose, with the remaining ST was still in free form (total recovery 81%). After 57 days the fructose and ST solution treated in a similar manner gave 21% bound ST and a 95% recovery. The glucose and ST solution was checked again after 9 months and was found to contain 85% of the glucose-bound ST with an 82% recovery of ST material. The ST and glucose solution maintained at room temperature for 9 months showed no change in total UV absorbance at 280 nm (in 3.5 N hydrochloric acid) compared with the original starting absorbance.

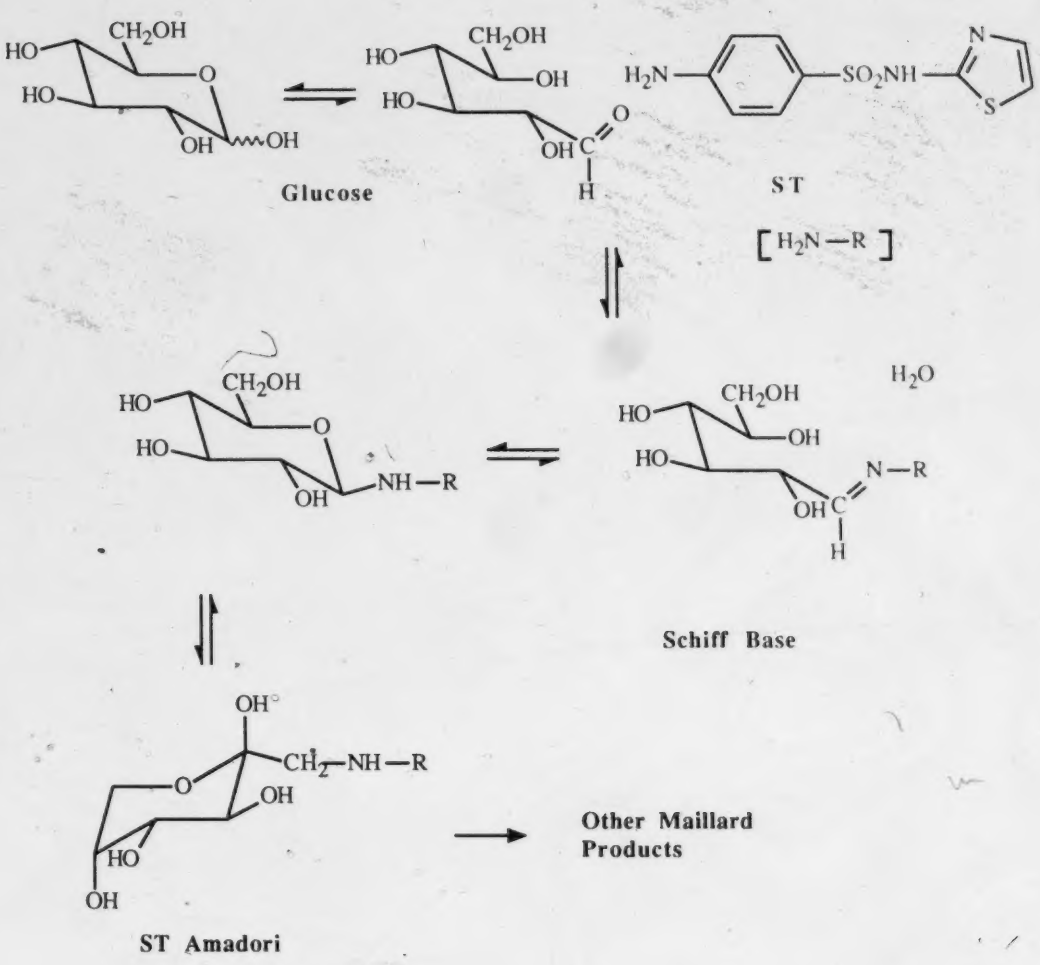
An experiment was carried out with a small amount of ST (100 ppm) and the same concentration of glucose as before. Even though glucose was present in this reaction at almost a 5000-fold molar excess compared with ST, periodic examination by TLC did not indicate significantly faster formation of glucose-bound ST than earlier solutions containing only a threefold excess of glucose.

Further confirmation of the ST-sugar reaction was provided by independent synthesis of the 1-(N^4-sulfathiazolyl)-1-deoxy-D-fructose ST Amadori compound, (Scheme 6). The ST Amadori compound had an R_f of 0.21, absorbed UV light, could be charred, and did not react with the B-M spray. Therefore, it seemed that at least some of the material

formed in the ST and glucose solution and present in the TLC streak was the ST Amadori compound.

If isolated glucose-bound ST-containing no free ST was diluted in water, TLC examination indicated the formation of free ST from the glucose-bound material. The ST Amadori also produced some free ST after aqueous dilution but at a considerably slower rate. If either material was diluted in 3.5 N hydrochloric acid, 85-90% of the bound ST was almost immediately converted to the free form as judged by the amount of color development in a B-M reaction (Low *et al.*, 1989). Leaving either material for long periods of time in the acid solution did not increase the amount of color development. TLC examination of the either of the bound ST materials after acid treatment also indicated a large increase in free ST. Since quantitation of free ST formed under these acid conditions depended on the amount of color formed in the B-M reaction, it could not be ruled out that the part of the reason that such a large proportion of the bound material was converted to free ST was that free ST was removed as it reacted further through the diazonium salt to the colored compound. As free ST reacted to form colored compound the ST-sugar reaction equilibrium would adjust to form more free ST. The fact that less than total recovery of free ST occurred under acid conditions could be due to formation of compounds such as N^4-acetyl-ST in reactions similar to those described by Giera *et al.* (1982) in sulfamethazine residue studies.

SCHEME 6



It was obvious from earlier work of Low *et al.* (1989) that when ST was heated in solution in the presence of reducing sugar, an increasing amount of ST was converted into sugar-bound form that could not be reversed to free ST in 3.5 N hydrochloric acid. To investigate further the conditions required to produce increasing amounts of irreversibly bound ST-sugar derivatives, four reactions containing 1000 ppm ST were prepared in the amount of glucose as before (1.75 M). These samples were heated at different temperatures, and the total amounts of free ST and glucose-bound ST that could be

reversed by acidic B-M reaction conditions (Low *et al.*, 1989) were quantitated. Aliquots of the reaction were treated with 3.5 N hydrochloric acid for about 30 s followed by quantitation using the B-M reaction (Table 2.1). As heating temperature increased, the rate of formation of irreversibly bound ST-glucose derivatives increased. Maillard browning (Waller and Feather, 1983) was obvious as a result of heating as all solutions turned yellow and later brown. Since none of the solutions was buffered, the reaction was complicated by the fact that acidic products of the reaction lowered the pH as the reaction progressed. Therefore the reaction at 80°C dropped from pH 8.2 to pH 3.9 in 187 h. Similar, although slower, decreases in pH were observed in other solutions. The UV absorbance of these reactions at 280 nm in 3.5 N hydrochloric acid was also checked and no decrease in absorbance was seen. After a time (for example, about 48 h with the 80 ^{0}C reaction) there was an increase in absorbance at 280 nm due to the formation of UV-absorbing products from the Maillard reaction. When a portion of the concentrated ST and glucose reaction mixture stored at room temperature for 9 months was tested with the B-M reaction, it was found that about 72% of the ST could be detected. This decrease in B-M colored material occurred despite the fact that all of the ST was detected by using UV absorbance at 280 nm, as noted above.

From these observations, the reaction of ST with glucose could be summarized as shown in Scheme 6. ST reacted with the reducing sugar to give a Schiff base, which could further react to form the ST Amadori compound. Some of the glucose bound ST, possibly the Schiff base material, could rapidly form free ST again on aqueous dilution. Other glucose-bound ST material, such as the ST Amadori compound formed free ST less rapidly under these conditions. Under strongly acidic conditions, the equilibria between all of these compounds favored formation of the free ST due to protonation of the aromatic amino group (ST has a p*Ka* of 2.4; Bell and Roblin, 1942). Eventually, after a long period of storage at low temperatures, or a shorter time at elevated temperatures, compounds such as the ST Amadori compound formed later Maillard products. These products, which include

Table 2.1. Temperature Effect on Sulfathiazole in an Aqueous Glucose Solution* Monitored Using a Bratton-Marshall Colorimetric Assay.

Temperature	Time (h) for Starting Absorbance (545nm) to Decrease by Half
80°C	104
67°C	187
62°C	240
50°C	245

* 1000 ppm sulfathiazole in 1.75 M aqueous glucose solution

polymeric brown material, bind ST more strongly, and significant amounts of ST could not be freed by acid treatment.

The effect of heating sulfamethazine residues in meat was studied by Epstein *et al.* (1988) and O'Brien *et al.* (1981); however, it is not clear from these papers whether "losses" of sulfamethazine can be attributed to reaction with reducing sugars.

The significance of ST reacting with reducing sugars in sulfonamide residue analysis is that all of the sugar bound sulfonamide residues (virtually all sulfonamides have the free reactive aromatic amine group) have different chromatographic mobilities, have different solubilities, and do not show up with a commonly used B-M spray on TLC. Different chromatographic mobilities of sugar bound sulfonamides have different retention times in HPLC. Therefore, unless these peaks are taken into considerations; these quantities of sulfonamides are missed. Some of the sugar-bound sulfonamides can act as depots for sulfonamide, releasing free sulfonamide after dilution.

Since a variety of other methods are used for sulfonamide analyses besides chromatographic and colorimetric methods, it would also be useful to understand the effect that sugar-bound sulfonamides have on other methods of analysis.

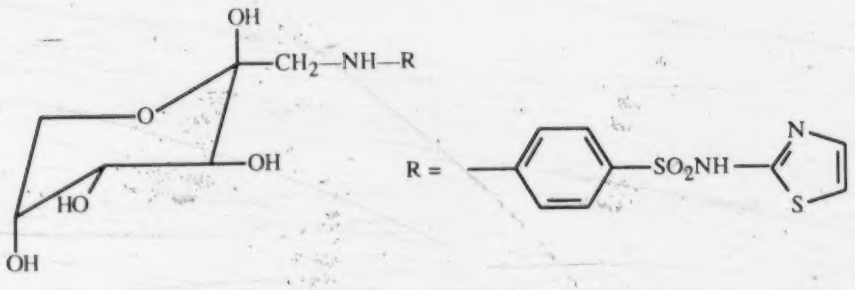
2.3.2 Effect of Reducing Sugars Reacting with ST on Microbiological Analysis Methods

The earliest methods of sulfonamide residue analysis in food included microbiological inhibition tests. For example, Read *et al.* (1971) described a method widely used to detect sulfonamides and other antibiotics in milk. Starting with two bacterial organisms that were susceptible to ST, *Escherichia coli* ATCC 11775 and *Staphylococcus aureus* ATCC 25923, we found that the minimum inhibitory concentrations of ST were 1 and 3 μg (in 10 μL of solvent), respectively. When ST Amadori, succinyl ST (Figure 2.1), purified glucose-bound ST, or purified fructose-bound ST was applied up to 100 μg (in 10 μL of solvent), neither organism was inhibited. Not only will traditional microbial inhibition tests not detect sugar-bound ST or any N^4-substituted sulfonamides but similar

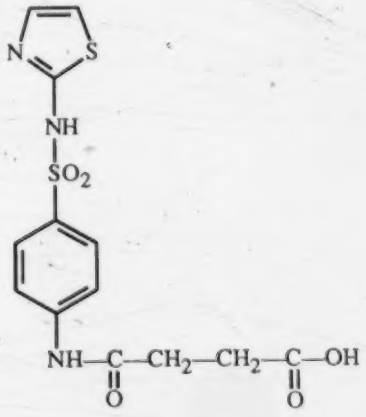
tests based on microbial receptors, such as Charm II test, will almost certainly suffer from the same problems since the microbial receptor recognizes the free amino group on the sulfonamide (Charm and Chi, 1988). Of course, these microbial tests are rarely used for quantitation of sulfonamide residues. If sufficient free sulfonamide or, during workup, sufficient sugar-bound sulfonamide is converted to free sulfonamide, a microbial test would detect sulfonamide residues.

2.3.3 Effect of Reducing Sugars Reacting with ST on ELISA Methods

Recently, researchers such as Fleeker and Lovett (1985), Dixon-Holland and Katz (1988) and Singh *et al.* (1989), and a number of commercial companies (CITE, Sulfamethazine Test Kit, Agritech Systems Inc., 100 Fore St, Portland, MN 04101; EZ-SCREEN Sulfamethazine Test, Environmental Diagnostics, Inc., Box 908, 2990 Anthony Road, Burlington, NC 27215; Sulfamethazine Immunoassay Test Kit, Idetek, Inc., 1057 Sneath Lane, San Bruno, CA 94066) have introduced immunoassay based procedures for sulfamethazine residue analysis. To investigate the effect that sugar-bound ST compounds would have on this type of test, an indirect competitive ELISA was developed for ST. As seen from the data in Table 2.2 (structures given in Figure 1.1), rabbit serum was very specific for ST and related compounds. A 10% reduction of absorbance was noted with concentrations of only 10 ppb ST. To reduce the absorbance by half required concentrations of about 100 ppb (3.6×10^{-2} nM/assay) ST. Sulfonamides with any group other than a thiazole R group attached to the sulfonamide residue did not compete effectively for antibody. The best sulfonamide competitors containing different R groups were sulfapyridine and sulfadiazine, which, at 500-fold increase in concentration, reduce the final absorbance by half. SuccinylST was expected to compete effectively for antibody and on a weight basis proved to be as effective as ST. Since succinylST has a higher molecular weight than ST, this meant that on a molar basis, succinylST was a better competitor than ST.



ST Amadori



Succinyl ST

Figure 2.1. ST Amadori and Succinyl ST.

Table 2.2. Competitive Indirect ELISA of Sulfonamides Using Polyclonal Rabbit Serum.

Compound	Concentration of Compound (nM/assay) Required to Reduce Absorbance of Control by Half
ST	3.6×10^{-2}
ST Amadori	1.2×10^{-3}
succinyl ST	2.8×10^{-2}
sulfapyridine	20.06
sulfadiazine	19.98
sulfamerazine	>34.93
sulfamethazine	>33.30
sulfamethizole	>37.00
sulfisoxazole	>37.41
sulfacetamide	>46.69
sulfadimethoxine	>30.09
sulfamoxole	>37.41
sulfanilamide	>58.07

Surprisingly, on a weight basis, the ST Amadori compound was 20 times as effective a competitor for antibody as ST. On a molar basis, the ST Amadori compound was about 33 times as effective. When the structure of the ST-LPH conjugate used to obtain the antibodies was considered, however, this increase in sensitivity of the antibodies to ST Amadori compound was explained. The conjugate most likely contained most of the azo ST compound linked to tyrosine residues as shown in Scheme 4. The antibodies developed with this conjugate obviously bound much better to the more similar Amadori ST compound than to ST itself.

The effect of sulfonamides bound to reducing sugars in ELISA-type analyses of sulfonamides can therefore have dramatic effects on the detection limit of these tests. Although there are different methods of preparing sulfonamide protein conjugates (Dixon-Holland and Katz, 1988), all antisera developed, including antisera used in commercial sulfamethazine kits, are specific for the R group of the sulfonamide. This means that the sulfonamide has been linked to the protein through the aromatic amino group (N^4 amino group). The aromatic amino group is also the easiest portion of the molecule to attach to a protein. Antisera developed against such antigens would probably bind better to N^4-derivatized sulfonamides. Fleeker and Lovett (1985) did test the sulfamethazine "metabolites", acetylsulfamethazine and glucosylsulfamethazine. They found that the acetylsulfamethazine bound better, but the glucosyl derivative bound less to antisera than sulfamethazine. We did not test either isolated glucose-bound ST or fructose-bound ST (which contained compounds similar to the glucosyl derivative) in our ELISA procedure, since these mixtures rapidly produced free ST under the aqueous ELISA conditions. Fleeker and Lovett (1985) also noted the instability of glucosylsulfamethazine under acidic aqueous conditions. Although their glucosylsulfamethazine was synthesized under nonaqueous conditions, the stability of this compound under the aqueous conditions of their ELISA experiments was not noted. There is, however, the potential for all ELISA-type methods to have considerably lower detection levels for sugar-bound sulfonamides

than for free sulfonamides (Sheth and Sporns, 1990), and even low levels of sugar-bound sulfonamides could dramatically effect quantitation.

Overall then, if samples are to be analyzed for sulfonamide residues, the samples should be analyzed as rapidly as possible, especially if the samples are known to contain reducing sugars. If storage is required, refrigeration is recommended to limit the conversion of free sulfonamide to the sugar-bound form. Some of the sugar-bound material can be made to release sulfonamide again using an acid step in the sample workup. Also, if sulfonamide reaction with reducing sugars in a sample is suspected, the occurrence of this reaction can be confirmed by initially heating a portion of the sample. The heated sample will give lower sulfonamide residue values than an unheated sample in all cases, except for ELISA-type methods, where the reverse can occur.

2.3.4 Problems involved with ST Determination Methods in Honey

Some variation of the B-M test is often used to screen honey samples for ST. In the B-M test, the aromatic amino portion of the sulfonamide compound develops a pink color through a diazo reaction with a suitable naphthalene derivative. Honey samples can differ a great deal in color, however, and the interpretation of the color of a honey reacted directly with B-M reagents is difficult at best.

An alternative is to extract honey and use the extract for B-M detection. We investigated such a modified B-M test, using 10 g of honey A (defined in experimental). The honey sample was diluted with 10 mL of 1.0 N hydrochloric acid and extracted with 3 portions of 20 mL ethyl acetate. The ethyl acetate was removed using an evaporator and a modified B-M test (Low *et al.*, 1989) was performed on the residue. Absorbance at the optimum wavelength (545 nm) was found to be 0.38 (or about 2 ppm ST equivalent for the original honey sample). Because this honey was known to be free of ST, it was obvious that other compounds in honey could also develop a pink color using the B-M test. Other studies (Bregha-Morris, 1979) have reduced or eliminated B-M interference of compounds

in honey by pH adjustments and a series of extractions; however, the procedures then become so lengthy and use such large quantities of honey that they are limited in their utility for screening honey samples for ST.

ST has been concentrated from honey using an anion exchange resin before screening for ST using a modified B-M test (Schwartz and Sherma, 1986). AG MP-1 anion exchange resin (100-200 mesh) was prepared as suggested by Schwartz and Sherma (1986). Several columns were prepared using 1 mL resin with 1 mL 20 ppm ST added. The ST remained on the column as judged by examination of elute with B-M analysis. Next, replicate attempts were made to elute the column-bound ST with 6 N and 12 N hydrochloric acid. Recoveries of ST in acid eluates as determined by the B-M test were 2.0 and 2.5%, respectively. Schwartz and Sherma used only very small amounts of resin in their procedure. It was likely that enough ST leaked from the small amount of resin to allow a colorimetric indication of ST. Although ST binds very strongly to the resin, from our results, it can not be quantitatively removed even with very strong acid. Therefore, Schwartz and Sherma's procedure for screening honey for ST is dependent on the amount of resin used and could miss honey samples contaminated with ST. These findings were also confirmed in another laboratory [N.H. Low personal communication].

It is not practical to screen ST in honey on a routine basis using chromatographic methods such as liquid chromatography, or TLC because of the time and cost involved, so we worked to develop an EIA method for screening honey samples for ST.

2.3.5 Enzyme Immunoassay for Screening Honey

When different ST-free honey samples were diluted 4 times (w/v) with water and analyzed using the EIA procedure, great variation in background absorbances was observed. To overcome this background interference, likely due to high concentrations of sugar in the samples (known to be free of ST), these same honey samples were diluted 30,

times (w/v) and tested using the EIA procedure. Table 2.3 shows absorbance values (at 450 nm) of 5 different honey samples in triplicate. These absorbance values were not statistically different (P <0.05).

To test the effect of ST on EIA absorbance, 2 ST free honey samples were diluted 30 times with ST added so that final concentrations of ST were 0, 10, 50, 75, and 100 ppb. These samples were carried through the EIA procedure and absorbance recorded. Because it was known that ST reacts with the reducing sugars in honey (Sheth *et al.*, 1990) and that this reaction affected all methods of quantitation of sulfonamides, 1 honey sample was also subjected to heating at 80 ^{0}C for 62 h. These temperature conditions were known to form both reversibly and irreversibly sugar-bound ST (Sheth *et al.*, 1990). The absorbance data for these experiments are given in Table 2.4. All 3 experiments were carried out on different days using different microtiter plates. There was considerable day-to-day and plate-to-plate variation in final absorbance values. Honey samples containing ST, however, were consistently lower in absorbance and the absorbance decrease was proportional to the amounts of ST present.

To standardize values for comparison, we decided to examine the data as absorbance ratios with the average absorbance value at any ST concentration (B) divided by the absorbance of diluted honey containing no ST (Bo). Now the effect of ST content on the honey samples was easier to compare (Table 2.5). Even though the absorbance values for the 2 unheated honey samples were quite different, the B/Bo values were similar. Also, the heated honey sample behaved as expected (Sheth *et al.*, 1990), with Maillard reaction ST products generally binding antibody better than free ST, resulting in lower B/Bo values. Honey samples containing at least as little as 0.3 ppm ST (i.e. 10 ppb for honey diluted 30-fold) could be detected with the EIA procedure.

Table 2.3. EIA Absorbances at 450 nm of Honey Samples After Thirty-Fold Dilution.

Honey Samples*				
A	B	C	D	E
1.372	1.401	1.445	1.402	1.449
1.312	1.328	1.367	1.379	1.385
1.370	1.321	1.411	1.362	1.339
1.351 ± 0.034	1.350 ± 0.044	1.410 ± 0.039	1.381 ± 0.020	1.391 ± 0.055

* final row contains average values ± standard deviation

Table 2.4. EIA Absorbances at 450 nm of Honey Samples With ST Added.

ST concentration (ppb)	Honey Samples		
	D*	E*	E, heated at 80°C for 62 h*
0	1.691	1.062	1.171
	1.721	1.047	1.119
	1.845	1.119	1.271
	1.807	1.144	1.269
	1.583	1.107	(1.208 ± 0.075)
	(1.729 ± 0.103)	(1.096 ± 0.040)	
10	1.474	0.842	0.766
	1.483	0.902	0.734
	1.655	1.010	0.803
	1.549	1.064	0.840
	1.475	1.029	(0.786 ± 0.046)
	(1.527 ± 0.078)	(0.969 ± 0.093)	
50	1.134	0.817	0.349
	1.189	0.790	0.347
	1.364	0.796	0.385
	1.309	0.886	0.416
	1.258	0.868	(0.374 ± 0.033)
	(1.251 ± 0.092)	(0.831 ± 0.043)	
75	1.161	0.703	0.307
	1.145	0.748	0.321
	1.254	0.791	0.363
	1.230	0.840	0.357
	1.133	0.804	(0.337 ± 0.027)
	(1.185 ± 0.054)	(0.777 ± 0.053)	
100	0.926	0.632	0.263
	0.965	0.685	0.231
	1.054	0.713	0.243
	1.082	0.674	0.280
	1.062	0.734	(0.254 ± 0.022)
	(1.018 ± 0.068)	(0.688 ± 0.039)	

* average values in brackets ± standard deviation

Table 2.5. Data from Table 2.4 presented as Percentage Absorbance Ratios (B/B_O).

ST concentration (ppb)	Honey Samples		
	D	E	E, heated at 80°C for 62 h
10	88.3	88.4	65.1
50	72.4	75.8	31.0
75	68.5	70.9	27.9
100	58.9	62.8	21.0

One of the main advantages of the EIA procedure for screening honey samples was that very small amounts of honey were required. Because the microtiter plates contained 96 wells and required only 100 µL of 30 times diluted honey per well, numerous replicates and/or samples could be examined simultaneously. This made the screening of honey very efficient. The EIA described here could be used, to screen not only for ST, but also roughly quantitate honey samples for ST because the amount of absorbance decreased proportionally to the amount of ST present.

2.3.6 Comparison of Honey Analysis by EIA with the Results of Other Laboratories

The EIA procedure was used to examine honey check samples provided by the Audit and Method Verification Unit of Agriculture Canada's Laboratory Service Division. These check samples were used by all Canadian laboratories accredited for ST analysis of honey. It was felt that this double blind sample set would fully test the rigors of our EIA, because we expected some honey samples to contain ST, and some to be free of ST. Table 2.6 shows our replicate quantitative results (run on different plates) compared with the average quantitation from 5 accredited laboratories using all methods at their disposal (mainly liquid chromatography). As the results show, samples, 1-1, 2-2; 1-2, 2-3; and 1-3, 2-1; respectively, were in fact duplicate samples and although the EIA predicted it, to our surprise, all honey samples contained ST. Generally, our quantitation values were higher than those of other laboratories and this could be explained in part by the fact that we were unable to do the analyses immediately and had to store samples frozen for 1 month. The original handling of samples, plus our added delay, may have caused increased sugar-bound ST, which is known to decrease quantitation by other methods, while increasing quantitation of the EIA procedure (Sheth *et al.*, 1990).

Table 2.6. Honey Check Sample Results for ST (ppm).

Sample #	EIA Results	Accredited Laboratories' Results*
1-1	1.9	1.80 ± 0.38
	2.4	
1-2	1.1	1.06 ± 0.21
	1.7	
1-3	2.4	0.50 ± 0.38
	2.7	
2-1	2.6	0.50 ± 0.38
	2.8	
2-2	2.0	1.80 ± 0.38
	2.9	
2-3	0.7	1.06 ± 0.21
	0.6	

* ± standard deviation

We find that EIA procedure is a very efficient method for screening honey samples for ST. Simple dilution of honey is the only preparation necessary. Color or other interferences from honey were not a problem and sample throughput was rapid because 96 tests could be performed simultaneously on 1 microtiter plate. Furthermore, using B/Bo values, a rough quantitation of ST in the honey samples was also possible.

2.4 Sulfonamide Group Recognition

2.4.1 ELISA with 3a-Protein conjugates

Sulfonamides are synthetic antibiotics, the majority of which have the basic structure shown at the top of Figure 1.1 (Introduction). The simplest preparation of immunizing conjugate for a specific sulfonamide, involves linking through the aromatic amino group common to all sulfonamides. With this linkage the presentation of the hapten is such that the antibodies mainly develop against the R group which is farthest from the protein. Therefore these antibodies are excellent for the recognition of individual sulfonamides, but since the antibodies are sensitive to minor changes in the R group, they will not recognize other closely related sulfonamides. If an antibody that is cross-reactive with many sulfonamides is desired, the aromatic amino group must be free and the hapten constructed so that attachment to the protein is through R group.

Antibody was produced in two pairs of rabbits, which were immunized with 3a conjugated LPH and 3a conjugated BSA (Scheme 5). Indirect competitive ELISA tests performed on collected sera using 3a-OV as the coating conjugate resulted in high titers in each pair of rabbits. Serum dilution of 1/200,000 gave three times background absorbance. However, when the sera were tested in an indirect competitive ELISA with high concentrations (25 ppm for all sulfonamides, 10 ppm for 3a) of different commercial sulfonamides, all with free aromatic amino groups, no significant decrease in absorbance was noted when compared with the control. That is, free sulfonamides at high concentrations could not compete with bound hapten 3a. To confirm that this lack of

competition was not due to high avidity of the antibody molecules, Fab fragments were prepared. Use of Fab fragments in the assay resulted in no improvement in competition.

When 3a-Protein conjugates were prepared, 3a was linked to protein through an ε amino group of lysine. From the results, it seemed that during the immune response antibodies were produced against 3a and the terminal portion of lysine. The majority of these antibodies had greater affinity for this entire structure than for other sulfonamides; even free 3a. Another factor was that at the pH of the ELISA experiments, the acid group of free 3a was negatively charged, unlike the 3a conjugates. This phenomenon of linking arm effect has also been noted by other researchers (Vallejo *et al.*, 1982; Wie and Hammock, 1984; Harrison *et al.*, 1991).

In order to select the subpopulation of antibodies with specificities for the aromatic amino portion of sulfonamides, 3d-OV and base hydrolyzed 5-OV conjugates were prepared (schemes 5 and 4). These conjugates differed from the immunogens in R group and, especially with base hydrolyzed 5-OV in the linking arm portion. Serum obtained from 3a-BSA immunizing conjugate did not have antibody subpopulations for the base hydrolyzed 5-OV conjugate, judging by ELISA experiments (only background absorbance noted). However, when serum from 3a-LPH immunogen was tested with base hydrolyzed 5-OV coating conjugate, eleven sulfonamides (Table 2.7) were able to reduce absorbance in the indirect competitive ELISA. Sulfonamides with smaller R groups (sulfacetamide and sulfanilamide) and with larger R groups (sulfamethazine and sulfamerazine) were poorly or undetected in the assay. An exception seemed to be sulfadimethoxine which contained a lot of steric bulk in the R group but was detected by ELISA procedure. It was interesting to note that considerable electronic variation in the R group was tolerated by the antibodies, but steric considerations less tolerated. As expected, the antibodies were most sensitive to 3a.

Table 2.7. Concentration (nM/assay) of Sulfonamide required to reduce the ELISA Absorbance by Half.

Sulfonamide	3a conjugated LPH immunogen with base hydrolyzed 5 conjugated OV in ELISA	3a conjugated BSA immunogen with 3d conjugated OV in ELISA	3a conjugated LPH immunogen with 3d conjugated OV in ELISA
3a	0.006	0.14	0.64
3b	0.33	0.28	>8.5
Sulfapyridine	0.50	1.2	>10.0
Sulfamethizole	0.47	0.22	0.47
Sulfadiazine	0.64	2.8	>10.0
Sulfamethoxazole	1.3	2.8	>9.9
Sulfamoxole	1.4	2.6	>9.4
Sulfadimethoxine	1.5	2.6	8.1
ST	1.8	0.14	62.2
Sulfacetamide	8.4	>11.7	>11.7
Sulfanilamide	12.5	>14.5	>14.5
Sulfamerazine	>9.0	>9.0	>9.0
Sulfamethazine	>8.7	>8.7	>8.7

Antibodies from both groups of rabbits immunized with 3a-LPH and 3a-BSA bound to 3d-OV (Table 2.7). In the case of 3a-LPH immunogen, only three sulfonamide structures were competitive, while with 3a-BSA immunogen nine sulfonamide structure were competitive.

When 3a-OV was used for coating ELISA plates, 1/10,000 of serum gave an absorbance of about 1.0 O.D. after a 30 minute substrate incubation. As expected, fewer antibodies were bound to the modified (base hydrolyzed 5 and 3d) coating conjugates and at least 10 fold more concentrated sera (1/500 with base hydrolyzed 5-OV and 1/1000 with 3d-OV) was required to achieve similar absorbances. However, from these results it was obvious that by proper choice of immunizing hapten, coating hapten and protein linking procedure, ELISA methods could be developed with broad sulfonamide group recognition.

2.4.2 ELISA with 7-LPH Serum

As described above except sulfadimethoxine, sulfonamides with bulk in their R groups (sulfamethazine and sulfamerazine) were not detected by 3a-LPH and 3a-BSA sera. Some of the sulfonamides with bulky R groups (such as sulfaquinoxaline, sulfamerazine and sulfamethazine) are very important because they are under strict regulatory requirements. Therefore immunizing conjugates were prepared with bulk in their R groups (3e-LPH, 3f-LPH). Indirect competitive ELISA was performed using 3e-LPH and 3f-LPH sera in combination with 5 different coating conjugates (3a-OV, base hydrolyzed 5-OV, 3d-OV, 3e-OV and 3f-OV). None of the sulfonamides was able to compete with protein bound haptens in coating conjugates.

After these failures compound 7 was synthesized, linked to LPH, used as an immunogen, and serum was obtained. As in previous experiments 3a-OV, 3d-OV and base hydrolyzed 5-OV conjugates were used for coating microtiter plates. Antibodies from this serum did not bind to base hydrolyzed 5-OV coating conjugate and only background absorbance was observed. Free sulfonamides were unable to compete with protein linked 3d in

3d-OV coating conjugate. However, when compound 7-LPH serum was tested with 3a-OV conjugate 14 out of 16 sulfonamides were detected. As expected, sulfonamides with smaller R groups (sulfanilamide and sulfacetamide) were not detected at lower concentrations (sulfanilamide up to 58.07 nM/assay and sulfacetamide up to 11.67 nM/assay).

Figure 2.2 shows the B/Bo values versus concentrations of sulfadimethoxine for 3a-BSA and 7-LPH sera while Figure 2.3 shows the B/Bo values versus concentrations of sulfamethizole for 3a-LPH and 7-LPH sera. It is evident from these Figures that 3a-LPH and 3a-BSA sera required very low concentrations of sulfonamides to get B/Bo = 0.5. High background absorbance was observed in indirect competitive ELISA when 7-LPH serum was used. This serum detected 14 sulfonamides at lower concentrations, however higher amounts of sulfonamides were required to obtain B/Bo = 0.5. If these later results are reported as B/Bo = 0.5 (like in 3a-BSA and 3a-LPH sera) values for all sulfonamides, misleading conclusions are likely to be drawn. Because of this it was decided to present B/Bo values for each concentration of all sulfonamides (Tables 2.8-2.15).

In all experiments competitive indirect ELISA was done using polyclonal sera. As name indicates these sera contained heterogeneous population of antibodies and results obtained from these sera are gross effects of all populations of antibodies. In this type of sera different populations of antibodies have different affinities for different ligands. In case of 3a-BSA and 3a-LPH sera, entire population of antibodies which bound to coating conjugates and could also be dislodged by free sulfonamides. In case of 7-LPH serum some antibodies were sensitive to low concentrations of free sulfonamides which unbound from the coating conjugate, there were some antibody populations which required higher concentrations of free sulfonamides.

From these experiments it was clear that sterically hindered sulfonamides which could not be detected by 3a-LPH and 3a-BSA sera were detected by the 7-LPH sera (with greater bulk in R group) and therefore it was able to detect sulfamethazine, sulfamerazine and sulfaquinoxaline.

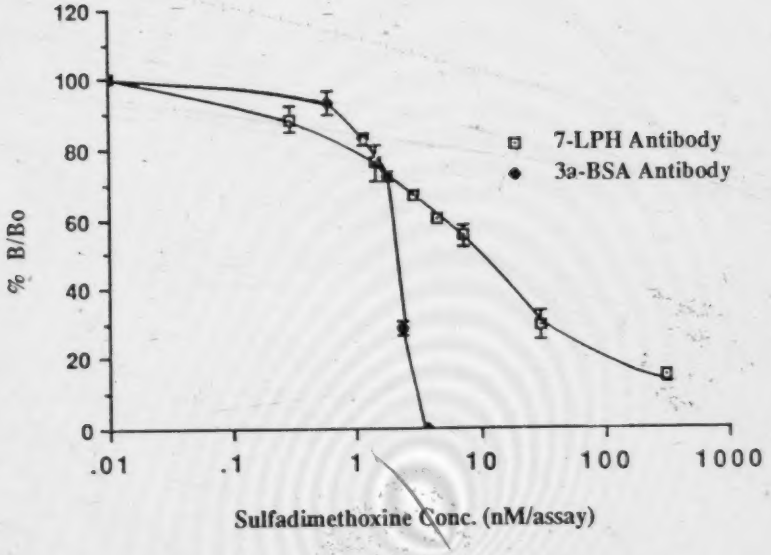


Figure 2.2 % B/Bo Vs. Sulfadimethoxine Conc.

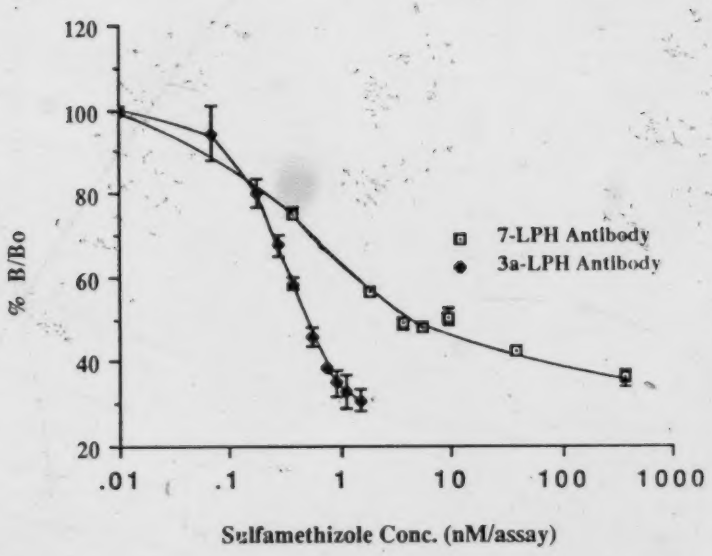
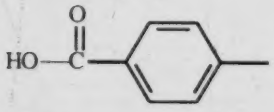
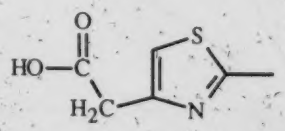


Figure 2.3 % B/Bo Vs. Sulfamethizole Conc.

Table 2.8. B/Bo Values of 3d and 3a.

3d			3a		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.34	92.9	5.2	0.32	79.5	3.8
1.71	86.2	4.7	1.60	61.3	5.0
3.43	78.5	5.1	3.20	48.6	3.1
5.14	82.0	3.6	4.79	43.9	3.3
8.56	74.7	2.5	7.99	48.3	1.1
342.5	18.3	17.5	319.4	14.6	9.0
Bo = 1.37, C.V. = 3.0			Bo = 1.37, C.V. = 3.0		

R = 

R = 

The above "R" groups and those in Tables 2.9-2.15 are moieties on the sulfonamide structure:

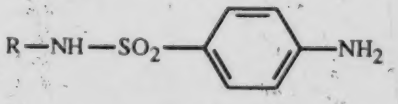
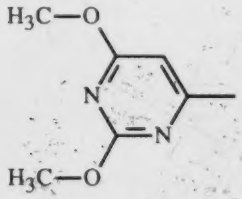


Table 2.9. B/Bo Values of Sulfadimethoxine and ST.

Sulfadimethoxine			ST		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.30	88.4	4.4	0.36	93.2	6.0
1.51	75.7	6.5	1.80	70.4	5.0
6.02	66.4	1.1	3.61	58.9	5.2
3.01	59.7	1.8	5.41	51.3	7.0
7.52	54.8	5.4	9.02	46.0	4.7
30.09	29.3	13.9	36.06	40.6	3.4
300.9	14.8	10.8	360.6	42.1	9.3
Bo = 1.37, C.V. = 3.0			Bo = 1.37, C.V. = 3.0		

R = 

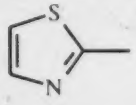
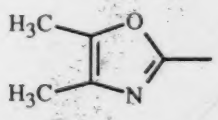
R = 

Table 2.10. B/Bo Values of Sulfamoxole and Sulfamethizole.

Sulfamoxole			Sulfamethizole		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.37	94.2	4.6	0.37	75.3	2.1
1.87	78.2	5.4	1.85	56.6	1.0
3.74	79.5	4.7	3.70	49.1	2.8
5.61	70.1	1.6	5.55	48.2	1.5
9.35	76.1	7.4	9.25	50.6	4.3
37.41	55.2	5.0	37.00	42.2	2.3
374.1	3.9	3.1	370.0	36.1	6.0
Bo = 1.46, C.V. = 4.3			Bo = 2.04, C.V. = 7.8		

R = 

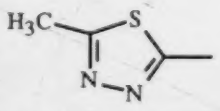
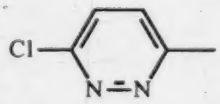
R = 

Table 2.11. B/Bo Values of Sulfachloropyridazine and Sulfameter.

Sulfachloropyridazine			Sulfameter		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.35	64.1	3.1	0.36	85.0	3.5
1.76	48.6	2.8	1.78	73.5	3.8
3.51	44.2	7.0	3.57	60.8	2.3
5.27	41.8	1.3	5.35	57.3	2.2
8.78	44.0	3.4	8.92	53.4	2.3
35.12	40.7	6.3	35.67	42.9	2.3
351.2	6.3	8.6	356.7	26.4	2.9
Bo = 2.04, C.V. = 7.8			Bo = 2.04, C.V. = 7.8		

R = 

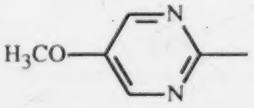
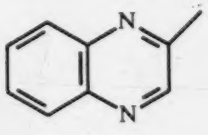
R = 

Table 2.12. B/Bo Values of Sulfaquinoxaline and Base Hydrolyzed Sulfanitran.

Sulfaquinoxaline			Base Hydrolyzed Sulfanitran		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.31	88.5	4.7	0.34	85.1	2.6
1.55	78.1	1.5	1.71	72.6	5.0
3.10	72.4	0.4	3.41	72.2	0.4
4.65	69.0	3.1	5.12	67.4	1.6
7.76	68.9	3.7	8.53	60.1	6.8
--	--	--	34.13	53.6	4.3
310.3	24.0	4.1	341.3	5.8	1.0
Bo = 2.04, C.V. = 7.8			Bo = 2.02, C.V. = 5.1		

R = 

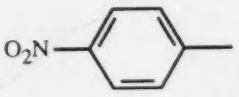
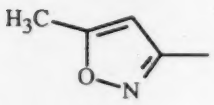
R = 

Table 2.13. B/Bo Values of Sulfamethoxazole and Sulfamerazine.

Sulfamethoxazole			Sulfamerazine		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.40	87.6	1.8	0.35	81.8	4.5
1.97	67.6	1.5	1.75	76.4	9.8
3.95	69.1	4.2	3.49	63.3	4.2
5.92	62.9	1.6	5.24	59.3	7.8
9.87	57.7	1.5	8.73	55.1	8.4
39.47	52.0	1.1	34.92	36.8	6.8
394.7	41.7	4.2	349.3	31.0	6.6
Bo = 2.02, C.V. = 5.1			Bo = 1.08, C.V. = 2.3		

R = 

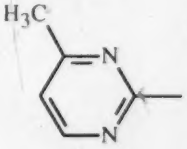
R = 

Table 2.14. B/Bo Values of Sulfacetamide and Sulfanilamide.

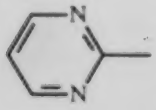
Sulfacetamide			Sulfanilamide		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.47	98.9	2.6	0.58	94.3	2.2
2.33	96.9	4.2	2.90	94.4	0.9
4.67	103.3	2.9	5.8	101.5	3.2
7.00	102.6	2.1	8.71	101.9	6.1
11.67	99.9	1.7	14.52	98.7	4.7
46.69	76.9	5.7	58.07	96.5	3.7
466.9	34.0	4.5	580.7	11.2	15.5
Bo = 2.02, C.V. = 5.1			Bo = 2.02, C.V. = 5.1		

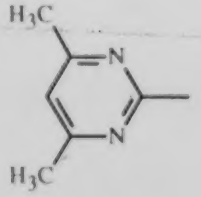
R = $H_3C-\overset{\overset{O}{\|}}{C}-$

R = H—

Table 2.15. B/Bo Values of Sulfadiazine and Sulfamethazine.

Sulfadiazine			Sulfamethazine		
nM/Assay	% B/Bo	C.V.	nM/Assay	% B/Bo	C.V.
0.40	89.4	1.8	0.33	95.3	6.6
2.00	72.5	6.4	1.67	87.4	3.5
4.00	63.1	2.7	3.33	91.5	6.2
5.99	57.1	3.0	5.00	77.1	6.3
9.99	57.9	5.8	---	---	---
39.95	45.4	3.6	33.30	73.4	5.3
399.5	35.9	2.5	333.0	42.4	8.8
Bo = 1.46, C.V. = 4.3			Bo = 1.46, C.V. = 4.3		

R = 

R = 

3. EXPERIMENTAL

3.1 Materials

Water was prepared using a Millipore Milli-Q system. For flash chromatography 230-400 mesh silica gel (E. Merck, Darmstadt, Germany) was used. FCA, FIA, tryptic soy agar and MH broth were obtained from Difco Laboratories (Detroit, MI). MH agar was supplied by Oxoid Ltd., Busingstoke, England. 2-Amino-4-thiazole acetic acid, N-acetyl sulfanilyl chloride, *p*-nitroaniline, N-hydroxy succinimide, 1,3-dicyclohexyl carbodiimide and N,N-dimethylformamide were purchased from Aldrich (Milwaukee, WI). All sulfonamides, thimerosal, Tween 20, BSA, LPH and OV were obtained from Sigma Chemical Co. (St. Louis, MO). Platinum dioxide was supplied by BDH Inc. (Edmonton, AB). Urea peroxide, *o*-phenylenediamine tablets, goat anti-rabbit peroxidase conjugated antibodies and citric acid (monohydrate) were obtained from Calbiochem Co. (San Diego, CA). Immobilized papain and protein A were supplied by Pierce (Rockford, IL). Dynatech Immulon 1 microtiter plates and Spectrapor dialysis tubing (12,000-14,000 M.W. cutoff) were purchased from Fisher Scientific (Edmonton, AB). TLC plates were 20 x 20 cm PE SIL G/UV with polyester backing obtained from Whatman Ltd. (Maidstone, England). Filter papers GVWP-025 were purchased from Millipore Corp. (Bedford, MA.). Ion exchange resin (100-200 mesh), AG MP-1, chloride form was obtained from Bio-Rad Laboratories, Richmond, CA. Honey sample A was obtained from a local, noncommercial beekeeper who did not use any chemicals. Other honey samples, free of ST (samples B through E) were courtesy of the Food Laboratory Services Branch, Alberta Agriculture (a laboratory accredited by Agriculture Canada for ST analysis in honey).

3.2 Instrumentation

Absorbance values at 280 nm and 545 nm were recorded with a Hewlett-Packard Model 8451A diode array spectrophotometer (Hewlett-Packard [Canada] Ltd., Mississauga, ON). Microtiter plate optical densities were measured with a Model EL 309 ELISA reader (Bio-Tek Instruments, Inc., Burlington, VT). Buchi Rotavapor-R (Fisher Scientific,

Edmonton, AB) was used for the removal of solvents from samples. A Mastermite Model 10008 heat gun (Master Appliance Corp., Racine, WI) was used to dry TLC plates. A model UVS - 54 lamp (254-nm light emission produced by Ultraviolet Products, Inc., San Gabriel, CA) was used to check for TLC UV absorbance. Centrifugation was performed with a Damon/IES division Model HN-S II centrifuge from International Equipment Co. (Needham Heights, MA). Fraction collector, FRAC-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used to collect fractions in the IgG isolation and in the generation of Fab fragments. Econo-pac 10 mL disposable polypropylene column (Bio-Rad, Richmond, CA.) was connected to FRAC-100 fraction collector in the isolation of IgG. Virtis freeze dryer (Virtis Company Inc., Gardiner, N.Y.) was used to dry protein conjugates and IgG. Reichert Thermovar (Vienna, Austria) was used to measure the melting points of crystalline materials. TLC was used to monitor the progress of chemical reactions. Spots were visualized and/or stained with B-M reagents (Parks, 1982). No effort was made to optimize the yields of the reactions.

NMR analysis was carried out by high field NMR Laboratory of Chemistry Services at the University of Alberta. NMR spectra were measured on WH-200, AM-300 or WH-400 instruments. Compounds used for NMR analysis were either crystalline or gave one spot on TLC.

MS were obtained using a Kratos AEI MS-50 (high resolution, electron impact ionization) for exact mass determination. MS analysis was done by the Mass Spectrometry Laboratory of Chemistry Services at the University of Alberta. Fragmentation pattern of all significant peaks and peaks with more than 20% intensity was recorded and explained. Only mass spectral peaks greater than 100 were considered. When it was not possible to explain the fragmentation of a specific peak with more than 20% intensity, it was recorded as:

X (Y) UE

where X = mass of the peak, Y = peak intensity, and UE = unexplained

3.3 Buffers

PBS

Sodium chloride (18.0 g), disodium hydrogen phosphate (2.215 g), potassium dihydrogen phosphate (0.600 g) and thimerosal (0.200 g) were dissolved in a little less than 2 L of water. The pH was adjusted to 7.3 with concentrated sodium hydroxide. The adjusted solution was made up to 2 L with Milli-Q water. PBS for the isolation of IgG contained 0.02% of sodium azide but no thimerosal.

Tween 20 (0.05 % w/v) was added to PBS to obtain PBST.

Digestion buffer

Digestion buffer was prepared just prior to use. Sodium dihydrogen phosphate (2.76 g), sodium tetraethylenediamine tetraacetate (3.80 g) and cysteine hydrochloride (3.51 g) were taken into 1L volumetric flask and dissolved in approximately 950 mL of water. The pH of the solution was adjusted to 7.0 with 1 M sodium hydroxide and volume was made up to 1 L.

3.4 Concentrated Sugar-ST Reactions

Two solutions were prepared, one using glucose and one using fructose. The respective reducing sugars (78.8 g, 0.437 mol) and 37.2 g (0.134 mol) of sodium ST were added to a 250 mL volumetric flask with water and dissolved, and the flask was made up to volume with water. A magnetic stir bar was added to each flask, and each solution was stirred at room temperature protected from light with aluminum foil.

Total free and bound ST, or isolated free and isolated bound ST, were estimated by dissolving samples in 3.5 M hydrochloric acid and reading the absorbance at 280 nm (extinction coefficient 11700 M^{-1} cm^{-1}).

Free and bound ST could be separated by silica gel chromatography. The sugar-ST solution (0.5 mL) was added to 20 mL of water and 1 g of silica gel. Water was removed with a flash evaporator. The dried material was applied to the top of a 15 g silica gel column (2.2 cm i.d. x 30 cm height) and free ST eluted with ca. 200 mL of 1:4 methanol-

chloroform until no further UV-absorbing material could be detected by TLC. The sugar-bound ST was eluted with 1:1 methanol-chloroform (ca. 150 mL). Pooled samples of each material were evaporated to dryness for use in other experiments.

3.5 Microbial Growth Inhibition Test

All sugars and broths were sterilized before use at 121°C for 15 min in an autoclave. The test organisms, from the American Type Culture Collection, were either *Escherichia coli* (ATCC 11775) or *Staphylococcus aureus* (ATCC 25923). They were maintained on a tryptic soy agar slant at 4°C and subcultured twice in Mueller Hinton (MH) broth at 35°C for 18-24 h for use in the experiments.

Petri dishes (150-mm diameter) were prepared by adding 70 mL of MH agar and drying for 30 min in a laminar flow hood. A "soft" MH overlayer (21 g/L MH broth and 7.5 g/L MH agar) was sterilized and tempered to 45°C. MH broth containing one of the organisms was added to the "soft" MH overlay agar (10 µL/mL agar) and mixed gently. The prepared "soft" MH overlayer was added to the perti dishes (17 mL/dish) and dried for 30 min at room temperature in a safety hood. Solutions (10 µL) of known amounts of ST, succinyl-ST, and ST Amadori in water or isolated glucose-bound ST and fructose-bound ST in methanol (10 µL of methanol alone did not inhibit microbial growth) were spotted with a micropipet onto the surface of the MH overlayer. The spots were separated by at least 4 cm. The petri dishes were dried until the spots disappeared in a safety hood. The petri dishes were inverted, placed into a plastic bag, and incubated for 18-24 h at 35°C. Clear zones of inhibition, if present, were observed to determine minimum inhibitory concentrations of compounds.

3.6 Preparation of Honey Samples with varying ST Concentrations

Dissolve ST-free honey (3.3 g) in ca 20 mL water, filter into 100 mL volumetric flask using filter paper No. 42 (Whatman, Clifton, NJ) and make up to volume with water. Pipet diluted honey into 5 different test tubes, 5 mL/tube, and add concentrated aqueous ST

solution to honey solutions in test tubes to give 0, 10, 50, 75, and 100 ppb standards respectively.

For the temperature study, the same type of test tube set was prepared and maintained at 80°C for 62 h.

3.7 Isolation of IgG from rabbit serum

An Econo-Pac 10 mL disposable polypropylene column was connected to a bench top fraction collector FRAC-100 and 0.6 mL protein A slurry was loaded. The column was then washed with 10 mL of PBS. Filtered rabbit serum (0.5 mL) was slowly added on top of the protein A column and allowed to sit for 15 minutes. First approximately 15 mL of PBS was used to elute proteins other than IgG at the rate of 1 mL/3 min. These serum proteins were collected in 1 mL fractions and their presence was determined by monitoring at 278 nm. The column was washed with PBS until no UV activity was noticed at 278 nm. Acetic acid (1M) was used to elute IgG. Fractions 2, 3, 4 and 5 were transferred into dialysis tubing and dialyzed against 4 L of 25 mM ammonium bicarbonate for 24 h with changing of solution every 4 h. The contents of the dialysis bag were freeze dried and stored at -20°C. Recovery of IgG from 0.5 mL of rabbit serum was 2 mg.

3.8 Generation of Fab fragments

Immobilized papain was gently mixed and 0.5 mL was added to a glass test tube. Digestion buffer (4 mL) was then added to the test tube and enzyme was separated from buffer by centrifugation. This procedure of washing enzyme with digestion buffer was repeated one more time and immobilized papain was resuspended in 0.5 mL of digestion buffer.

Lyophilized IgG (4 mg) was dissolved in 2 mL of fresh digestion buffer and added to the test tube containing prepared papain. The enzyme-IgG mixture was incubated for five hours in a shaker water bath at 37°C. After 5 h of incubation, immobilized papain was separated from the digest by centrifugation. Prior to centrifugation, 1.5 mL of 10 mM Tris

HCl, pH 7.5, was added to the IgG-enzyme digest. The Fab fragments were separated from unhydrolyzed IgG and Fc fragments using the protein A column. The mixture of Fab fragments, unhydrolyzed IgG, and Fc fragments was loaded on top of protein A slurry and the column was washed with 10 mL of PBS at the rate of 1 mL/3 min. Fractions 2, 3 and 4 were transferred into dialysis tubing and dialyzed against 4 L of 25 mM ammonium bicarbonate for 24 h, changing the solution every 4 h. The contents of the dialysis bag were freeze dried and stored at -20 ^{0} C. Recovery of Fab from 0.5 mL of rabbit serum was 1.1 mg.

3.9 Chemical Synthesis

3.9.1 Synthesis of 1-(N^4-ST)-1-deoxy-D-fructose (ST Amadori)

This compound was synthesized by Dr. V. Yaylayan (Sheth *et al.*, 1990) of the Macdonald College of McGill University. ^{1}H NMR (60 MHz, D_2O), δ (1.6 (2H, CH_2N), 3.0-3.6 (5H, fructose), 6.1-7.5 (6H, Aromatic).

3.9.2 Synthesis of 2-amino-4-thiazole acetic acid methyl ester (compound # 1a, synthesis #1)

Methanol (100 mL), previously dried over anhydrous sodium sulfate was added to a 250 mL three-necked flask in an ice bath. Hydrochloric acid gas was bubbled into the flask, while the methanol was being stirred until the weight of the solution had increased by about 60 g. 2-Amino-4-thiazole acetic acid (2.1 g, 13.29 mM) was slowly added to the flask and the solution was removed from the ice bath and stirred for another 10 min. The contents of the flask then subjected to reflux conditions for 6 h, resulting in a slightly yellow solution. All solvent was removed on a flash evaporator, dry methanol (25 mL) added, and this solvent also removed. The resulting solid was recrystallized (melting point 169-171°C) from ethyl acetate and methanol to give 2-amino-4-thiazole acetic acid methyl ester as slightly yellow crystals (1.9 g, 83.1% yield): ^{1}H NMR (400 MHz, DMSO-d_6), δ

9.30 (brs, 2H, -N*H*), 6.71(s, 1H), 3.76(s, 2H, -C*H*$_2$), 3.65 (s, 3H, -C*H*$_3$). Exact mass M^+, m/z 172 (87.29 %) calcd for $C_6H_8N_2O_2S$, 172.0306 (measured 172.0308); $(M-CH_4O)^+$, 140 (10.52%), $C_5H_4N_2OS$, 140.0044 (140.0045); $(M-C_2H_2O_2)^+$, 114 (20.35%), $C_4H_6N_2S$, 114.0252 (114.0235); $(M-C_2H_3O_2)^+$, 113 (100%), $C_4H_5N_2S$, 113.0173 (113.0161).

3.9.3 Synthesis of p-amino benzoic acid methyl ester (compound #1d)

p-Amino benzoic acid methyl ester was synthesized from 10.0 g (72.97 mM) of *p*-amino benzoic acid using the synthesis #1. Yield of *p*-amino benzoic acid methyl ester was 9.41 g (85.3%). ^{1}H NMR (200 MHz, DMSO-d_6), δ 8.46 (brs, 2H, -N*H*), 7.83 (d, 2H, 7.60, Ar-*H*), 7.05 (d, 2H, 7.60, Ar-*H*), 3.79 (s, 3H, -C*H*$_3$). Exact mass M^+, m/z 151 (58.01 %) calcd for $C_8H_9NO_2$, 151.0633 (measured 151.0632); $(M-CH_3O)^+$, 120 (100%), C_7H_6NO, 120.0449 (120.0450).

3.9.4 Synthesis of N^4-acetyl-N^1-[4-(methoxycarbonyl)methyl-2-thiazolyl] sulfanilamide (compound # 2a, synthesis # 2)

N-Acetyl sulfanilyl chloride (2.65 g, 11.34 mM) was added to a 50 mL round bottom flask and 5 mL dry pyridine was added with stirring. 2-Amino-4-thiazole acetic acid methyl ester (1.50 g, 8.72 mM) was added to the N-acetyl sulfanilyl chloride-pyridine solution slowly and maintained at a temperature of not more than 40°C. The brownish solution was refluxed for 1.5 h and allowed to cool to room temperature. While stirring the solution vigorously, warm water (30 mL) was added and the mixture extracted with (3 x 25 mL) methylene chloride. The combined methylene chloride layers were dried over sodium sulfate, filtered and most of the solvent removed on a flash evaporator. About 5 g of silica gel was added and the remaining solvent removed to leave a yellowish powder which was applied to a 50 g silica column (50 x 2.4 cm). The column was eluted with 1:4 methanol:chloroform (150 mL). Removal of solvent gave the N^4-acetyl-N^1-[4-(methoxycarbonyl)methyl-2-thiazolyl]sulfanilamide (1.60 g, 49.69% yield) ^{1}H NMR (300

MHz, DMSO-d_6), δ 12.35 (brs, 1H, -N*H*), 10.25 (s, 1H, -N*H*), 7.70 (s, 4H, Ar-*H*), 6.60 (s, 1H), 3.65 (s, 2H, -C*H*$_2$), 3.61 (s, 3H, -C*H*$_3$), 2.05 (s, 3H, -C*H*$_3$). Exact mass $(M\text{-}CH_4O)^+$, m/z 337 (1.20%) calcd for $C_{13}H_{11}N_3O_4S_2$, 337.0191 (measured 337.0202); $(M\text{-}C_2H_2O_2)^+$, 311 (2.37%), $C_{12}H_{13}N_3O_3S_2$, 311.0398 (311.0395); $(M\text{-}SO_2)^+$, 305 (10.32 %) $C_{14}H_{15}N_3O_3S$, 305.0834 (305.0835); $(M\text{-}C_2H_2O_4S)^+$, 247 (3.30%), $C_{12}H_{13}N_3OS$, 247.0779 (247.0769); $(M\text{-}C_6H_7N_2O_2S)^+$, 198 (16.15%), $C_8H_8NO_3S$, 198.0225 (198.0239); $(M\text{-}C_8H_6NO_3S)^+$, 173 (15.09%), $C_6H_9N_2O_2S$,173.0385 (173.0357); $(M\text{-}C_8H_7NO_3S)^+$, 172 (94.35%), $C_6H_8N_2O_2S$, 172.0306 (172.0306); $(M\text{-}C_8H_9N_2O_3S)^+$, 156 (9.13%), $C_6H_6NO_2S$, 156.0119 (156.0118) $(M\text{-}C_9H_{11}NO_4S)^+$, 140 (33.35%), $C_5H_4N_2OS$, 140.0044 (140.0056); $(M\text{-}C_6H_6N_2O_4S)^+$, 135 (7.91%), C_8H_9NO, 135.0684 (135.0676); $(M\text{-}C_{11}H_9NO_5S)^+$, 114 (42.96%), $C_4H_6N_2S$, 114.0252 (114.0245); $(M\text{-}C_{11}H_8NO_5S)$+, 113 (100%), $C_4H_5N_2S$, 113.0173 (113.0158); $(M\text{-}C_8H_9N_2O_4S_2)$, 108 (8.51%) C_6H_6NO, 108.0449 (108.0451).

3.9.5 Synthesis of N^4-acetyl-N^1-(4-nitrophenyl)sulfanilamide (compound # 2b)

N^4-Acetyl-N^1-(4-nitrophenyl)sulfanilamide was synthesized from 7.0 g (50.71 mM) of *p*-nitroaniline and 20 g (85.59 mM) of N-acetyl sulfanilyl chloride using the synthesis #2. Yield of the product was 9.30 g (54.7%). Exact mass M^+, m/z 335 (36.03%), calcd for $C_{14}H_{13}N_3O_5S$, 335.0576 (measured 335.0574); $(M\text{-}C_6H_4N_2O_2)^+$, 199 (10.57%), $C_8H_9NO_3S$, 199.0303 (199.0259); $(M\text{-}C_6H_5N_2O_2)^+$, 198 (100%), $C_8H_8NO_3S$, 198.0225 (198.0228); $(M\text{-}C_8H_7N_2O_3)^+$, 156 (30.11%), $C_6H_6NO_2S$, 156.0119 (156.0115); $(M\text{-}C_8H_7NO_3S)^+$, 138 (16.31%), $C_6H_6N_2O_2$, 138.0429 (138.0430); $(M\text{-}C_6H_5N_2O_4S)^+$, 134 (51.54%), C_8H_8NO, 134.0606 (134.0607); $(M\text{-}C_8H_7N_2O_4S)^+$, 108 (35.59%), C_6H_6NO, 108.0449 (108.0451); $(M\text{-}C_8H_6NO_5S)^+$, 107 (10.68%), $C_6H_7N_2$, 107.0609 (107.0611).

3.9.6 Synthesis of N^1-(4-carboxymethyl-2-thiazolyl)sulfanilamide (compound # 3a, synthesis #3)

Sodium hydroxide solution (25 mL, 2M) was added to 0.95 g purified (2.57 mM) N^4-acetyl-N^1-[4-(methoxycarbonyl)methyl-2-thiazolyl]sulfanilamide. The resulting mixture was kept under reflux conditions for 2 h and cooled to room temperature. pH was then adjusted to 4.0 with 6 N hydrochloric acid. The turbid solution obtained in this manner was extracted with (3 x 25 mL) ethyl acetate.The combined ethyl acetate extract was dried over sodium sulfate, filtered and solvent removed to yield (0.26 g, 32.3% yield) a brown solid. ^{1}NMR (300 MHz, DMSO-d_6), δ 12.20 (brs, 1H, -COO*H*), unable to assign δ value (s, 1H, -N*H*), 7.40 (d, 2H, 7.80, Ar-*H*), 6.54 (d, 2H, 7.80, Ar-*H*), 6.46 (s, 1H), 5.82 (brs, 2H, -N*H*), 3.48 (s, 2H, -C*H*$_2$). Exact mass $(M\text{-}CO_2)^+$, m/z 269 (13.60 %), calcd for $C_{10}H_{11}N_3O_2S_2$, 269.0293 (measured 269.0291); $(M\text{-}CO_4S)^+$, 205 (36.36%), $C_{10}H_{11}N_3S$, 205.0674 (205.0674), $(M\text{-}C_5H_5N_2O_2S)^+$, 156 (61.66%), $C_6H_6NO_2S$, 156.0119 (156.0119); $(M\text{-}C_5H_5N_2O_3S)^+$, 140 (3.46%), C_6H_6NOS, 140.0170 (140.0169); $(M\text{-}C_7H_5NO_4S)^+$, 114 (100%), $C_4H_6N_2S$, 114.0252 (114.0252); $(M\text{-}C_5H_5N_2O_3S)^+$, 108 (7.34%), C_6H_6NO, 108.0449 (108.0453)

3.9.7 Synthesis of N^1-(4-carboxyphenyl)sulfanilamide (compound # 3d)

N^4-Acetyl-N^1-(4-carboxyphenyl)sulfanilamide was synthesized from 4.0 g (26.49 mM) of *p*-amino benzoic acid methyl ester and 7.5 g (32.10 mM) of N-acetylsulfanilyl chloride using the synthesis # 2. This material (1.1 g, 3.16 mM) was then hydrolyzed with 2M sodium hydroxide as described in synthesis # 3. Yield of the product was 0.21 g (22.8%). ^{1}H NMR (300 MHz, DMSO-d_6), δ 12.68 (brs, 1H, -COO*H*), 10.44 (s, 1H, -N*H*), 7.90 (s, 2H, -N*H*), 7.78 (d, 2H, 7.00, Ar-*H*), 7.46 (d, 2H, 7.00, Ar-*H*), 7.17 (d, 2H, 7.00, Ar-*H*), 6.56 (d, 2H, 7.00, Ar-*H*). Exact mass M^+, m/z 292 (8.92 %), calcd for $C_{13}H_{12}N_2O_4S$, 292.0518 (measured 292.0520); $(M\text{-}SO_2)^+$, 228 (1.04%); $C_{13}H_{12}N_2O_2$, 228.0899 (228.0903); $(M\text{-}C_7H_6NO_2)^+$, 156 (21.05%), $C_6H_6NO_2S$, 156.0119

(156.0121); $(M\text{-}C_6H_5NO_2S)^+$, 137 (100%), $C_7H_7NO_2$, 137.0477 (137.0481); $(M\text{-}C_6H_6NO_3S)^+$, 120 (97.67%), C_7H_6NO, 120.0449 (120.0451).

3.9.8 Synthesis of 2-amino-6-methoxy-4-(methoxycarbonylmethyl)pyrimidine (compound # 1e)

Worrall's (1943) method was used to synthesize 2-amino-4-methoxycarbonylmethyl-6-hydroxypyrimidine from diethyl 1,3-acetone-dicarboxylate (10.0 g) and guanidine carbonate (8.9 g). Product (5.0 g) of this step was used as a starting material for the synthesis of 2-amino-4(methoxycarbonylmethyl)-6-chloropyrimidine using the method of Bycroft *et al.* (1971). This product (2.0 g) was in turn used as a starting material for the synthesis of 2-Amino-6-methoxy-4-(methoxycarbonylmethyl)pyrimidine by the method of Bycroft *et al.* (1971). ^1H NMR (300 MHz, $CDCl_3$), δ 6.07 (s, 1H, Ar-*H*), 4.92 (brs, 2H, -N*H*), 3.87 (s, 3H, -C*H*$_3$), 3.73 (s, 3H, -C*H*$_3$),3.54 (s, 2H, -C*H*$_2$).

3.9.9 Synthesis of N^1-(6-methoxy-4-carboxymethyl-2-pyrimidinyl)sulfanilamide (compound # 3e)

The compound N^4-acetyl-N^1-[6-methoxy-4-(methoxycarbonylmethyl)2-pyrimidyl]-sulfanilamide was synthesized from 2-amino-6-methoxy-4-(methoxycarbonylmethyl)-pyrimidine (200 mg, 1.02 mM) and N-acetyl sulfanilyl chloride (350 mg, 1.50 mM) using synthesis # 2. Base hydrolysis (2M sodium hydroxide, 10 mL) of this material (192 mg, 0.49 mM), as described in synthesis # 3 gave (60 mg, 36.2% yield) N^1-(6-methoxy-4-carboxymethyl-2-pyrimidinyl)sulfanilamide. Exact mass 288 (24.14%) UE; $(M\text{-}CO_4S)^+$, m/z 230 (100%), calcd for $C_{12}H_{14}N_4O$, 230.1168 (measured 230.1162); $(M\text{-}CHO_4S)^+$, 229 (61.31%), $C_{12}H_{13}N_4O$, 229.1089 (229.1087); $(M\text{-}C_2H_3O_4S)^+$, 215 (3.90%), $C_{11}H_{11}N_4O$, 215.0933 (215.0926); $(M\text{-}C_7H_8N_3O_3)^+$, 156 (4.10%), $C_6H_6NO_2S$, 156.0119 (156.0117); $(M\text{-}C_7H_8N_3O_4)^+$, 140 (3.31%), C_6H_6NOS, 140.0170 (140.0172); $(M\text{-}C_7H_5NO_4S)^+$, 139 (5.49%), $C_6H_9N_3O$, 139.0746 (139.0741); $(M\text{-}C_7H_6NO_4S)^+$

138 (6.42%), $C_6H_8N_3O$, 138.0667 (138.0669); 124 (21.89%) UE; $(M-C_7H_8N_3O_4S)^+$, 108 (16.20%), C_6H_6NO, 108.0449 (108.0452).

3 9.10 Synthesis of N^1-(4-carboxymethyl-2-pyrimidinyl)sulfanilamide (compound # 3f)

Bycroft *et al.*'s (1971) method was used to synthesize 2-Amino-4-methoxy carbonylmethylpyrimidine from 2-amino-4-(methoxycarbonylmethyl)-6-chloropyrimidine (1.0 g). N^4-acetyl-N^1-(4-(methoxycarbonylmethyl)2-pyrimidinyl)sulfanilamide was synthesized from 2-Amino-4-(methoxycarbonylmethyl)pyrimidine (0.75 g, 4.49 mM) and N-acetyl sulfanilyl chloride (1.50 g, 6.42 mM) using synthesis # 2. Base hydrolysis (2M sodium hydroxide, 10 mL, synthesis # 3) of this material (850 mg, 2.34 mM) gave N^1-(6-methoxy-4-(methoxycarboxymethyl)2-pyrimidinyl)sulfanilamide (204 mg, 28.31% yield). Exact mass $(M-CO_4S)^+$, m/z 200 (86.34%) calcd for $C_{11}H_{12}N_4$, 200.1062 (measured 200.1054); $(M-CHO_4S)^+$, 199 (100%), $C_{11}H_{11}N_4$, 199.0984 (199.0985); $(M-CH_2NO_4S)^+$, 184 (2.40%), $C_{11}H_{10}N_3$, 184.0875 (184.0871); $(M-C_6H_6N_3O_2)^+$, 156 (7.20%), $C_6H_6NO_2S$, 156.0119 (156.0115); $(M-C_6H_6N_3O_3)^+$, 140 (3.63%), C_6H_6NOS, 140.0170 (140.0164); $(M-C_7H_4NO_4S)^+$, 110 (2.83%), $C_5H_8N_3$, 110.0718 (110.0674); $(M-C_7H_5NO_4S)^+$, 109 (39.69%), $C_5H_7N_3$, 109.0640 (109.0640); $(M-C_6H_6N_3O_3S)^+$, 108 (21.05%), C_6H_6NO, 108.0449 (108.0450).

3.9.11 Synthesis of N^4-acetyl-N^1-(4-methyl-5-nitro-2-pyridyl)sulfanilamide (compound # 2c)

This material was synthesized from 2-amino-4-methyl-5-nitro-pyridine (1.0 g. 6.49 mM) and N-Acetyl sulfanilyl chloride (2.3 g, 9.84 mM) employing synthesis # 2. Yield of the product was 1.3 g (57.3%). ^1H NMR (300 MHz, D_2O) δ 8.74 (s, 1H, Ar-*H*), 7.87 (d, 2H, 7.00, Ar-*H*), 7.66 (d, 2H, 7.00, Ar-*H*), 6.86 (s, 1H, Ar-*H*), 2.45 (s, 3H, -C*H*$_3$), 2.02 (s, 3H, -C*H*$_3$). Exact mass $(M-SO_2)^+$, 286 m/z (73.73%), calcd for $C_{14}H_{14}N_4O_3$, 286.1066 (measured 286.1053); $(M-HO_2S)^+$, 285 (100%), $C_{14}H_{13}N_4O_3$, 285.0988 (285.0986); $(M-C_2H_2O_3S)^+$, 244 (42.13%), $C_{12}H_{12}N_4O_2$, 244.0960 (244.0955); (M-

$C_2H_3O_3S)^+$, 243 (11.76%), $C_{12}H_{11}N_4O_2$, 243.0882 (243.0875); $(M\text{-}C_8H_8N_3O_3)^+$, 156 (6.57%), $C_6H_6NO_2S$, 156.0119 (156.0117); $(M\text{-}C_8H_7NO_3S)^+$, 153(5.20%), $C_6H_7N_3O_2$, 153.0538 (153.0541); $(M\text{-}C_8H_8N_3O_4)^+$, 140 (6.29%), C_6H_6NOS, 140.0170 (140.0168); $(M\text{-}C_6H_5N_3O_4S)^+$, 135 (3.28%), C_8H_9NO, 135.0684 (135.0641); $(M\text{-}C_6H_6N_3O_4S)^+$, 134 (29.69%), C_8H_8NO, 134.0606 (134.0605); $(M\text{-}C_8H_8N_3O_4S)^+$, 108 (19.15%), C_6H_6NO, 108.0449 (108.0449).

3.9.12 Synthesis of N^4-acetyl-N^1-(4-methyl-5-amino-2-pyridyl)sulfanilamide (compound # 6)

N^4-acetyl-N^1-(4-methyl-5-nitro-2-pyridyl)sulfanilamide (160 mg, 0.46 mM) was dissolved in 20 mL of methanol and platinum dioxide catalyst (160 mg) added to it. Hydrogen gas was injected into stirring sulfonamide-catalyst slurry for 18 h. Catalyst was then removed by filtration and the product (120 mg, 81.5% yield) was obtained by removing methanol. M^+, m/z 320 (7.39%), calcd for $C_{14}H_{16}N_4O_3S$, 320.0943 (measured 320.0937); $(M\text{-}SO_2)^+$, 256 (26.78%), $C_{14}H_{16}N_4O$, 256.1324 (256.1308); $(M\text{-}HO_2S)$+, 255 (49.06%), $C_{14}H_{15}N_4O$, 255.1246 (255.1242); $(M\text{-}C_6H_6N_2)^+$, 214 (11.60%), $C_8H_{10}N_2O_3S$, 214.0412 (214.0411); $(M\text{-}\ C_2H_3O_3S)^+$, 213 (10.01%), $C_{12}H_{13}N_4$, 213.1140 (213.1138); $(M\text{-}C_8H_8N_2O)^+$, 172 (36.17%), $C_6H_8N_2O_2S$, 172.0306 (172.0307); $(M\text{-}C_8H_{10}N_3O)^+$, 156 (18.41%), $C_6H_6NO_2S$, 156.0119 (156.0115); $(M\text{-}C_8H_{10}N_3O_2)^+$, 140 (4.86%), C_6H_6NOS, 140.0170 (140.0171); $(M\text{-}C_6H_7N_3O_2S)^+$, 135 (27.74%), C_8H_9NO, 135.0684, (135.0695); $(M\text{-}C_8H_7NO_3S)^+$, 123 (33.95%), $C_6H_9N_3$, 123.0796 (123.0788); $(M\text{-}C_8H_8NO_3S)^+$, 122 (100%), $C_6H_8N_3$, 122.0718 (122.0720); $(M\text{-}\ C_8H_8N_2O_3S)^+$, 108 (15.63%), $C_6H_8N_2$, 108.0687 (108.0687); $(M\text{-}C_8H_{10}N_3O_2S)^+$, 108 (14.27%), C_6H_6NO, 108.0449 (108.0450); $(M\text{-}C_8H_9N_2O_3S)^+$, 107 (16.82%), $C_6H_7N_2$, 107.0609 (107.0616); $(M\text{-}C_8H_9N_2O_3S)^+$, 107 (12.15%), $C_6H_7N_2$, 107.0609 (107.0572).

3.9.13 Synthesis of 2-amino-4,6-dimethyl-5-(4-nitrophenyl)azo-pyrimidine (compound # 4)

p -Nitroaniline (2.0 g) was dissolved in 8.0 mL of hot 6N hydrochloric acid. When *p*-nitroaniline was completely solubilized, the aqueous solution was transferred into an icebath. Sodium nitrite (45.4%, 2.2 mL) was added dropwise followed by the addition of 18 mL of 0.11% (v/v) acetyl acetone as described by O'Sullivan and Conalty (1980). Sodium acetate (3.5 N, 15 mL) was then added to the reaction mixture which gave yellow precipitate. This water washed percipitate (1.5 g) and guanidine hydrochloride (1.2 g) were dissolved in 15 mL of 3 N sodium hydroxide and 30 mL of methanol as described by O'Sullivan (1981). The resulting blood red solution was kept into bath maintained at 56°C for 20 h which gave rise to percipitation. Precipitate was filtered, washed (with 4 x 75 mL of 0.1 N hydrochloric acid followed by 2 x 100 mL of water) and dried in vacuum oven maintained at 50°C for 16 h. ^{1}H NMR (200 MHz, DMSO-d_6), δ 8.36 (d, 2H, 5.0, Ar-*H*), 7.91 (d, 2H, 5.0, Ar-*H*), 7.54 (brs, 2H, -N*H*), 3.35 (brs, 3H, -C*H*$_3$), 2.54 (brs, 3H, -C*H*$_3$). Exact mass $M^{+\cdot}$, m/z 272 (80.15), calcd for $C_{12}H_{12}N_6O_2$, 272.1022 (measured 272.1023); $(M\text{-}C_6H_4NO_2)^+$, 150 (61.63%), $C_6H_8N_5$, 150.0780 (150.0779); $(M\text{-}C_6H_6N_4)^+$, 138 (2.92%), $C_6H_6N_2O_2$, 138.0429 (138.0435); $(M\text{-}C_6H_4N_3O_2)^+$, 122 (100%), $C_6H_8N_3$, 122.0718 (122.0720).

3.9.14 Synthesis of N^1-[4-methyl-5-{2(4-carboxyethyl-1-hydroxyphenyl}azo)] sulfanilamide (compound # 7)

N^4-acetyl-N^1-(4-methyl-5-amino-2-pyridyl)sulfanilamide (82.2 mg, 0.26 mM) was dissolved in 2.5 mL of methanol and 2.5 mL of 1 N hydrochloric acid in a cold room. When solution was cold, 5 mL of 1 % sodium nitrite was added. This orange solution was stirred for 10 min. To neutralize excess of sodium nitrite, 4 mL of 0.8% ammonium sulfamate was added and the reaction was stirred for another 10 min. This reaction mixture was added to 3-(*p*-hydroxy phenyl)-propionic acid (50 mg, 0.30 mM) dissolved in 2 mL of

methanol. The pH of the solution was adjusted to 8.0 with 2M sodium hydroxide and the stirring reaction mixture was kept overnight at 4°C.

The next day solvent was removed using rotovaporator. Base hydrolysis, as described in synthesis # 3 of dried material was carried out for 30 min and after hydrolysis the solution was cooled to room temperature. The pH of the base hydrolyzed solution was adjusted to 4.0 with 1 N hydrochloric acid which gave dark brown precipitate (26 mg, 22% yield). This solid material was filtered, washed with 3 mL of milli-Q water and dried in vacuum oven at 40°C for 18 h. Exact mass $(M\text{-}C_6H_5NO_2S)^+$, m/z 300 (7.35%), calcd for $C_{15}H_{16}N_4O_3$, 300.1222 (measured 300.1222); $(M\text{-}C_9H_7NO_3)^+$, 278 (2.81%), $C_{12}H_{14}N_4O_2S$, 278.0837 (278.0831); $(M\text{-}C_9H_7NO_5S)^+$, 214 (30.65%), $C_{12}H_{14}N_4$, 214.1218 (214.1214); $(M\text{-}C_9H_8NO_5S)^+$, 213 (26.77%), $C_{12}H_{13}N_4$, 213.1140 (213.1141); $(M\text{-}C_9H_8N_2O_5S)^+$, 199 (68.41%), $C_{12}H_{13}N_3$, 199.1109 (199.1099); $(M\text{-}C_9H_9N_2O_5S)^+$, 198 (100%), $C_{12}H_{12}N_3$, 198.1031 (198.1031); $(M\text{-}C_{12}H_{10}N_4O_2S)^+$, 181 (20.17%), $C_9H_{11}NO_3$, 181.0739 (181.0739); $(M\text{-}C_{15}H_{13}N_3O_3)^+$, 172(10.20%), $C_6H_8N_2O_2S$, 172.0306 (172.0303); $(M\text{-}C_{15}H_{15}N_4O_3)^+$, 156 (21.57%), $C_6H_6NO_2S$, 156.0119 (156.0098); 149 (25.13%) UE; $(M\text{-}C_{15}H_{15}N_4O_4)^+$, 140 (8.61%), C_6H_6NOS, 140.0170 (140.0173); $(M\text{-}C_{15}H_{12}N_2O_5S)^+$, 123 (14.18%), $C_6H_9N_3$, 123.0796 (123.0785); $(M\text{-}C_{15}H_{13}N_2O_5S)^+$, 122 (74.08%), $C_6H_8N_3$, 122.0718 (122.0668); $(M\text{-}C_{15}H_{13}N_3O_5S)^+$, 108 (11.09%), $C_6H_8N_2$, 108.0687 (108.0687); $(M\text{-}C_{15}H_{15}N_4O_4S)^+$, 108 (31.59%), C_6H_6NO, 108.0449 (108.0452); $(M\text{-}C_{15}H_{14}N_3O_5S)^+$, 107 (14.13%), $C_6H_7N_2$, 107.0609 (107.0610); 107 (34.30%) UE; $(M\text{-}C_{14}H_{14}N_5O_4S)^+$, 107 (13.15%), C_7H_7O, 107.0497 (107.0497).

3.10 Synthesis of Sulfonamide-Protein Conjugates

3.10.1 Preparation of ST-BSA conjugate

Sodium ST (109 mg) was dissolved in 1.0 mL of 3.5 N hydrochloric acid in a 25 mL flask, and 3.0 mL of 1% sodium nitrite was added. This orange solution was stirred

for 10 min at room temperature. Ammonium sulfamate (50 mg) was added, and the reaction was stirred for another 10 min. This reaction mixture was added to BSA (202 mg) dissolved in 0.5 mL of water and 1.0 mL of 1.0 M sodium carbonate in a stirred 25 mL flask. A further 150 mg of sodium carbonate was added to the combined reaction, and the initial flask was washed with three portions of 0.25 mL of 1.0 M sodium carbonate. The washings were added to the stirring mixture, which was kept overnight at 4°C.

The next day the entire reaction mixture was transferred to dialysis tubing (6000-8000 MW cutoff) with 5 mL of 8.0 M urea and dialyzed against 1 L of 8.0 M urea, followed by 4 L of 50 mM ammonium bicarbonate and 4 L of 25 mM ammonium bicarbonate. Dialysis with each solution was carried out for a minimum of 8 h. The contents of the dialysis bag were freeze dried and submitted for elemental sulfur analysis.

3.10.2 Preparation of 3a-protein conjugates (synthesis # 4)

3a (62.5 mg), N-hydroxysuccinimide (34.5 mg) and 1,3-dicyclohexyl carbodiimide (45.3 mg) were reacted in 1.5 mL of dry N,N-dimethyl formamide at 4°C for 18 h. Dicyclohexyl urea precipitate produced in the reaction was removed by filtration. The filtrate was added to 65 mg LPH in 2 mL of PBS adjusted to pH of 7.6 with sodium hydroxide The mixture was stirred at 4°C for 24 h.

After 24 h, the reaction mixture was transferred to dialysis tubing and dialyzed against 1 L of 8 M urea for 12 h. The dialyzing solvent was then changed to 4 L of 50 mM ammonium bicarbonate followed by 4 L of 25 mM ammonium bicarbonate. Dialysis with each ammonium bicarbonate solution was carried out for a minimum of 8 h. The contents of the dialysis bag were freeze dried. 3a-BSA conjugate was produced using similar ratios of 3a, N-hydroxysuccinimide, 1,3-dicyclohexyl carbodiimide and BSA instead of LPH.

3.10.3 Preparation of base hydrolyzed 5-OV conjugate

N^4-acetyl-N^1-(4-aminophenyl)sulfanilamide (100 mg) was dissolved in 2 mL of 3.5 N hydrochloric acid in a 50 mL round bottom flask, and 4.5 mL of 1% sodium nitrite

added. The solution was stirred for 10 min at room temperature. Ammonium sulfamate (4.7 mL, 0.8 %) was added, and the reaction was stirred for another 10 min. This reaction solution was added to OV (200 mg) dissolved in 2.5 mL of PBS in a stirred 25 mL flask and the pH of the reaction adjusted to 9.0. The reaction mixture was kept at 4°C overnight with stirring.

Next day the reaction mixture was transferred to dialysis tubing and dialyzed as described above for the 3a-protein conjugates. The contents of the dialysis bag were freeze dried. This freeze dried protein conjugate was taken into a 100 mL round bottom flask, 10 mL of 2M sodium hydroxide added and subjected to reflux for 1h. After base hydrolysis the protein conjugate was dialyzed again, freeze dried and the epitope density (moles of base hydrolyzed sulfanitran/mole of protein) in the conjugate was determined.

3.10.4 Preparation of 3d-OV conjugate

3d-OV conjugate was prepared from 3d (35 mg) and OV (75 mg) by using the synthesis # 4. In this conjugate preparation after dialysis, dialyzed material was centrifuged at 8000 rpm for 1 h to separate insoluble particles. Supernatant was freeze dried and epitope density was determined.

3.10.5 Preparation of 3e-LPH conjugate

This conjugate was prepared from 3e (50 mg) and LPH (100 mg) using the synthesis # 4.

3.10.6 Preparation of 3f-LPH conjugate

This conjugate was prepared from 3f (30 mg) and LPH (60 mg) using the synthesis # 4.

3.10.7 Preparation of 7-LPH conjugate

This conjugate was prepared from 7 (40 mg) and LPH (65 mg) using the synthesis of #4.

3.11 Estimation of Epitope Density for Conjugates

The ST-BSA conjugate contained 4.51% sulfur, while BSA alone gave a sulfur analysis 1.68%. ST was found to have 19.82% sulfur because of moisture. From this analysis the calculated ST substitution in the ST-BSA conjugate was 44 per mol of BSA (66 000 MW). The ST-LPH conjugate contained 3.19% sulfur, while LPH alone gave a sulfur analysis 1.21%. Therefore the calculated ST substitution in the ST-LPH conjugate was 28 mol per BSA equivalent unit (i.e., using 66 000 MW so that comparison with the ST-BSA was convenient).

The presence of the free aromatic amino and group epitope density was measured by the modified B-M test of Low *et al.* (1989). Several sulfonamides (sulfadiazine, sulfapyridine, ST, and sulfamoxole) gave similar results on a mole basis and were used to determine the calibration curve for the aromatic amino group. LPH, BSA and OV gave no color in the modified B-M test. 3a-LPH, 3a-BSA, 3e-LPH and 3f-LPH used in immunization were insoluble under the modified B-M conditions (in large measure because of the number of group attached) but the insoluble protein particles developed an intense purple color indicating presence of a considerable number of free aromatic amino groups.

All conjugates used for ELISA coating were soluble in the above test and the number of moles of sulfonamide per mole of protein could be estimated. 3a conjugated OV had 10 moles hapten per mole protein; base hydrolyzed 5 conjugated OV had about 30 moles hapten per mole protein and 3d conjugated OV had about 5 moles hapten per mole protein.

3.12 Antibody preparation

Flemish Giant x Dutch Lop Ear cross rabbits were used for raising antibodies against different haptens. For the immunization of a pair of rabbits, 1 mg of immunogen was dissolved/suspended into sterile 1 mL PBS (without thimerosol) and 1 mL of FCA was added. The solution was thoroughly emulsified by forcing it back and forth in the syringe. Each rabbit was injected (2 x 0.2 mL) subscapularly and (0.4 mL) into a hip

muscle. The rabbits received identical booster injections, with the exception that FIA was used rather than FCA. Approximately two weeks after first booster injection rabbits were test bled from the large artery in the ear (approximately 15 mL from each rabbit). Rabbits were boosted until highest titer was obtained. When the desirable stage of titer was reached, rabbits were sacrificed and blood was collected by cardiac puncture. Collected blood was left for 1.5 h at room temperature, and the serum was decanted from the blood clot. The serum was centrifuged at 1000 rpm for 5 min, and the clear, yellowish serum, free of red blood cells, was collected into 1.5-mL sealed containers and stored at -20°C. Appendices 1-6 show the immunization regime of different immunogens.

3.13 Competitive Indirect ELISA Procedures

3.13.1 Competitive indirect ELISA procedure for screening sulfonamides using ST-LPH antibody

The 96 well microtiter plates were coated with 200 µL of 20 µg/mL BSA-ST in PBS per well.The plates were stored at 4°C overnight in a plastic bag containing a wet paper towel. The next day the solution was shaken from the plate and each well was coated with 200 µL of 1% BSA in PBS per well. The plates were again placed into the same plastic bag and held at room temperature for 1 h. The coating solution was shaken from the plate, and the plate was washed 3 times with PBST and blotted with force on paper tissues. Sulfonamides (in triplicate) at various concentrations including 0 concentration in PBS were added to wells (100 µL/well) followed by the addition of diluted serum (1/32 000 with 0.05% BSA in PBST) . The plate was held in plastic bag for 2 h. The antibiotic-antibody solution was removed from the plate and the plate was washed 3 times with PBST as before. Goat anti-rabbit peroxidase conjugated antibodies diluted 3000 times with PBST was added to each well (200 µL/well), including the controls, and the plate was stored in the plastic bag at room temperature for 2 h. The plate was washed 3 times with PBST as before. Substrates, *o*-phenylenediamine (0.4 mg/mL), and urea peroxide (1.0 mg /mL) in

0.1 M citrate buffer at pH 4.75 were added to each well (200 µg/mL). After exactly 30 min at room temperature the absorbance was measured with the ELISA reader set for 450 and 660 nm. Absorbance values for each well were recorded as the absorbance found at 450 nm minus the absorbance found at 660 nm.

3.13.2 Competitive indirect ELISA procedure for screening ST in honey

ELISA protocol used for the screening of ST in honey was the same as described above for the screening of sulfonamides with the exception that microtiter plates were coated with 200 µL/well of 0.1 µg/mL ST-BSA in PBS rather than 20 µg/mL. Also, instead of sulfonamides solutions honey samples (as described above) with ST (including no ST) were used in settting up the competition with the coating conjugate.

3.13.3 Competitive indirect ELISA procedure for screening sulfonamides using 3a-LPH or 3a-BSA antibody

ELISA protocol described above was used again with 3a-LPH and 3a-BSA sera with these changes. The 96 well microtiter plates were coated with 200 µL/mL of one of the coating conjugates (3a conjugated OV, 3d conjugated OV or base hydrolyzed 5 conjugated OV) in PBS. For different coating conjugates different dilutions of sera (serum from immunogen 3a conjugated BSA with ELISA coating 3a conjugated OV, 1/10,000; serum from immunogen 3a conjugated LPH with ELISA coating base hydrolyzed 5 conjugated OV 1/500; serum from immunogen 3a conjugated LPH or 3a conjugated BSA with ELISA coating 3d conjugated OV 1/1000) were used.

3.13.4 Competitive indirect ELISA procedure for screening sulfonamides using 7-LPH antibody

ELISA protocol described in 3.13.3 was used with the only change in the dilution of serum. A 1/100 dilution of 7-LPH serum was used in this procedure.

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5. APPENDICES

5.1 Immunization Regime of ST-LPH Immunogen

1) Description of Rabbits

a) Species: two female Flemish Giant x Dutch Lop Ear.

b) Age when immunized: 78 days

2) Immunization Protocol

Day 1: collection of blood and immunization with ST-LPH in FCA

Day 14: booster injection with ST-LPH in FIA

Day 28: test bleed and booster injection with ST-LPH in FIA

Day 42: collection of blood by cardiac puncture

5.2 Immunization Regime of 3a-LPH Immunogen

1) Description of Rabbits

a) Species: two female Flemish Giant x Dutch Lop Ear.

b) Age when immunized: 73 days

2) Immunization Protocol

Day 1: collection of blood and immunization with 3a-LPH in FCA

Day 14: booster injection with 3a-LPH in FIA

Day 28: test bleed and booster injection with 3a-LPH in FIA

Day 42: test bleed and booster injection with 3a-LPH in FIA

Day 56: test bleed and booster injection with 3a-LPH in FIA

Day 70: test bleed and booster injection with 3a-LPH in FIA

Day 82: collection of blood by cardiac puncture

5.3 Immunization Regime of 3a-BSA Immunogen

1) Description of Rabbits

a) Species: two female Flemish Giant x Dutch Lop Ear.

b) Age when immunized: 75 days

2) Immunization Protocol

Day 1: collection of blood and immunization with 3a-BSA in FCA

Day 28: booster injection with 3a-BSA in FIA

Day 38: test bleed and booster injection with 3a-BSA in FIA

Day 50: collection of blood by cardiac puncture

5.4 Immunization Regime of 3e-LPH Immunogen

1) Description of Rabbits

a) Species: one male and one female Flemish Giant x Dutch Lop Ear.

b) Age when immunized: 95 days

2) Immunization Protocol

Day 1: collection of blood and immunization with 3e-LPH in FCA

Day 28: booster injection with 3e-LPH in FIA

Day 41: test bleed and booster injection with 3e-LPH in FIA

Day 64: test bleed and booster injection with 3e-LPH in FIA

Day 77: collection of blood by cardiac puncture

5.5 Immunization Regime of 3f-LPH Immunogen

1) Description of Rabbit

a) Species: one male Flemish Giant x Dutch Lop Ear.

b) Age when immunized: 95 days

2) Immunization Protocol

Day 1: collection of blood and immunization with 3f-LPH in FCA

Day 28: booster injection with 3f-LPH in FIA

Day 41: test bleed and booster injection with 3f-LPH in FIA

Day 64: test bleed and booster injection with 3f-LPH in FIA

Day 77: collection of blood by cardiac puncture

5.6 Immunization Regime of 7-LPH Immunogen

1) Description of Rabbits

a) Species: two female Flemish Giant x Dutch Lop Ear.

b) Age when immunized: 77 days

2) Immunization Protocol

Day 1: collection of blood and immunization with 7-LPH in FCA

Day 21: booster injection with 7-LPH in FIA

Day 49: booster injection with 7-LPH in FIA

Day 63: test bleed and booster injection with 7-LPH in FIA

Day 77: test bleed and booster injection with 7-LPH in FIA

Day 91: test bleed and booster injection with 7-LPH in FIA

Day 105: test bleed and booster injection with 7-LPH in FIA

Day 119: collection of blood by cardiac puncture